ARCHIVES OF PATHOLOGY

VOLUME 33 FEBRUARY 1942 NUMBER 2

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# STUDIES OF CARTILAGE

## I. SOME EFFECTS OF MEDIUMS OF DIFFERENT $p_H$ VALUES ON THE COMPOSITION OF CARTILAGE

GEORGE M. HASS, M.D.*

AND

B. GARTHWAITE, A.B.

NEW YORK

Most intracellular and extracellular structures which can be visualized with the aid of the microscope have no definitions other than the ones provided by morphologists. These definitions lack no precision of description whenever form or changes in form are concerned, but they fail to satisfy inquiries which bear on the composition or the function of the structures in question. Some of these inquiries have been answered by resort to methods which provide descriptions of certain extractives in chemical nomenclature. These characterizations of total masses of tissue are of little value to morphologists. They do not specify the properties or the composition of unique microscopic structures.

It seems that a better understanding of these structures might be gained if a set of quantitative chemical and physical operations were applied arbitrarily to tissues and the effects of these operations on the tissues observed by morphologic methods.

This report is concerned with a study of human epiphysial cartilage by four standard chemical methods. The results of the study are presented and discussed from a chemical standpoint. The morphologic aspects will be correlated with the chemical data in a subsequent report.

### METHODS

The methods may be divided for discussion into five parts: (1) the preparation of cartilage; (2) the extraction of the prepared material; (3) the hydrolysis of the extracted tissue; (4) the determination of the quantity of reducing substances in the hydrolysate, and (5) the determination of the quantity of sulfate in the hydrolysate.

* Past member of the Society of Fellows, Harvard University.
From the Department of Pathology, Cornell University Medical College.

The method of preparing cartilage was as follows: The lower femoral and upper tibial epiphysial cartilages of infants were excised within a few hours after death. Ten infants from whom tissues were obtained were newborn. They varied from 1,700 to 4,000 Gm. in weight. The remaining 2 infants were 1 month and 9 months of age. The epiphysial cartilages were separated from the metaphyses by incisions in the plane of, and 2 mm. proximal to, the primary zones of ossification. The fresh blocks were then cut with a freezing microtome into sections which averaged 25 to 30 microns in thickness and 1 to 2 mg. in dry weight. The presence of ossification centers did not interfere with the preparation of sections, for the osseous tissue crumbled and broke away from the cartilaginous matrix everywhere except at the line of ossification.

The intact sections, usually 300 to 400 in number, were placed in a large funnel with a fritted glass disk and washed with repeated changes of distilled water at 5 C. over a period of six to eight hours. The sections were then divided equally into several lots, filtered over porous glass disks and transferred to weighed watch glasses. They were then dried in vacuo over sulfuric acid for twenty-four hours and subsequently to constant weight at 50 C. The difference in weights of the several lots of tissue varied about 5 mg. in any given experiment and from 35 to 80 mg. among the several experiments.

After constant weights had been obtained, each watch glass with adherent dry sections was placed in the aqueous solution which was to be used for extraction of that particular lot of tissue. Within thirty minutes the sections were thoroughly hydrated and with agitation were promptly dispersed as separate sections in each extractive medium.

Twelve complete controlled experiments were done with tissues prepared in this standard manner. The mediums used for extraction in complementary sets of experiments were as follows:

Two experiments: 1 per cent aqueous sodium chloride, $p_H$ 7; 10 per cent aqueous calcium chloride, $p_H$ 7; aqueous phosphate buffer solutions, $p_H$ 10 and $p_H$ 11.

Four experiments: aqueous two-tenths molar acetate buffer solutions, $p_H$ 4 and $p_H$ 5; 1 per cent aqueous sodium chloride, $p_H$ 7; 10 per cent aqueous calcium chloride, $p_H$ 7; phosphate buffer solutions, $p_H$ 10 and $p_H$ 11.

Six experiments: aqueous two-tenths molar acetate buffer solution, $p_H$ 5; aqueous phosphate buffer solution, $p_H$ 6; 1 per cent aqueous sodium chloride, $p_H$ 7; aqueous phosphate buffer solutions, $p_H$ 8.4, $p_H$ 10, $p_H$ 11 and $p_H$ 12.

The tissues were extracted for twenty-four hours at 5 C. in volumes of medium approximating 1 cc. per milligram of tissue. A few additional experiments were conducted to determine the effect of prolongation of the extraction period to five to seven days.

On termination of the period of extraction the sections invariably remained intact. They were filtered from the medium by use of fritted glass funnels and subsequently washed thoroughly with distilled water. After the washed sections had been partially dried by light suction, they were transferred to weighed watch glasses and after desiccation in vacuo were dried to constant weight at 50 C. The dried extracted tissues were then weighed and the loss of weight incidental to extraction computed.

For purposes of hydrolysis, 5 to 12 mg. of the dry extracted tissue was cut from each total mass immediately after obtaining the aforementioned weight. Considerable care was exercised in this operation and a quantity of tissue, large or small, depending on the expected low or high values for reducing substances and sulfate, was excised from each mass.

Preparatory to hydrolysis, these lots of dry tissue were transferred to separate pyrex tubes. Three cubic centimeters of four times normal hydrochloric acid was subsequently added to each tube containing experimental tissue, to one tube containing a standard control tissue and to one blank tube. The tubes were sealed in a flame and placed at 5 C. for twelve hours. Following this preliminary treatment at low temperature, the tubes were immersed in water at 100 C. for four hours. Then each tube was cooled, the sealed tip broken and the hydrolysate filtered into a graduated centrifuge tube. With appropriate washing of the hydrolysis tube and filter paper, the volume of each hydrolysate was brought up to 6 cc.

For the determination of reducing substances, 3 cc. of each of the hydrolysates, including the control and the reagent blank, was carefully neutralized with aqueous sodium hydroxide and brought up to a volume of 14 cc. with distilled water. After thorough mixing, 1 cc. was pipetted from each tube into a Folin sugar tube and made up to 4 cc. The determination of substances which reduce potassium ferricyanide was made thereafter according to Folin's method.[1]

For the determination of sulfate in the hydrolysates, including the control and the reagent blanks, 2 cc. was pipetted from each tube into a graduated centrifuge tube. These volumes were concentrated over a salt water bath at about 103 C. to 0.1 cc. and after being made up to 4 cc., or less when required, were centrifuged. From each volume, 1 cc. was removed and transferred to a centrifuge tube. For each experiment there were five tubes in addition to the ones containing experimental material: first, the tissue control; second, the reagent blank; third, the water blank; fourth, a standardization tube with 1 cc. of water containing 0.025 mg. of sulfate, and fifth, a standardization tube with 1 cc. of water containing 0.05 mg. of sulfate. From this point onward, the analysis was conducted by the micromethod of Cope.[2]

## RESULTS AND COMMENT

*Preparation of Tissue.*—The first method used in these studies provides fresh cartilage in thin uniform sections suitable for simultaneous morphologic and chemical manipulations. The sections are easily washed so that all water-soluble components, including red blood cells, are removed without producing any important morphologic change in the structure of the matrix or the cells of cartilage. The sections possess sufficient tenacity to withstand elementary manipulations incidental to dehydration, weighing, hydration, extraction and filtration. Further, the cartilage is in such form that serial observations may be made of morphologic effects of chemical operations up to the stage of complete disintegration of tissue structure. A final important advantage is that the method is adaptable to problems which require a topographic analysis of those regions of cartilage possessing a structure which varies from one microscopic field to another.

The principal fault of the method is the loss of water-soluble and highly unstable components during preparation of the sections. A second fault is the variable loss of weight of duplicate samples of tissue during washing, dehydration, hydration and filtration. No precautions

1. Folin, O.: New England J. Med. **206**:727, 1932.
2. Cope, C. L.: Biochem. J. **25**:1183, 1931.

were taken to correct the first fault, because highly stable cartilaginous residues were under investigation. The second fault, which might preclude use of the method in the study of many tissues, was a tolerable source of error in these experiments.

*Extraction of Tissue.*—The second procedure used in this study consists of an exposure of comparable lots of tissue to the action of buffer solutions possessing a wide range of $p_H$ values. The objective of the method is a controlled partial chemical dissection of the tissue. It has several advantages over the usual empiric technics of extraction with acid and alkaline reagents. One advantage is that the procedure is subject to accurate standardization. A second advantage is that the data which are obtained may be used for theoretic purposes, especially

TABLE 1.—*Percentages of Weight Lost by Twelve Samples of Cartilage During Extraction in Mediums with the Hydrogen Ion and Salt Concentrations Designated at the Heads of the Columns of Numbers*

| Experiment | $p_H$ 4 | $p_H$ 5 | $p_H$ 6 | $p_H$ 7 1% NaCl | $p_H$ 7 10% $CaCl_2$ | $p_H$ 8.4 | $p_H$ 10 | $p_H$ 11 | $p_H$ 12 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | ..... | ..... | ..... | 0.9 | 7.8 | ..... | 2.4 | 22.6 | ..... |
| 2 | ..... | ..... | ..... | 10.0 | 11.8 | ..... | 4.8 | 23.7 | ..... |
| 3 | 15.8 | 13.9 | ..... | 3.5 | 8.9 | ..... | 3.4 | 23.1 | ..... |
| 4 | 15.9 | 9.1 | ..... | 8.7 | 13.9 | ..... | 7.3 | 27.3 | ..... |
| 5 | 11.9 | 10.9 | ..... | 5.7 | 8.2 | ..... | 5.1 | 25.7 | ..... |
| 6 | 11.6 | 7.7 | ..... | 5.4 | 5.7 | ..... | 5.4 | 23.5 | ..... |
| Average * | **13.8** | **10.4** | ..... | **5.8** | **9.2** | ..... | **5.3** | **24.7** | ..... |
| 7 | ..... | 3.9 | 0.4 | 4.8 | ..... | 5.6 | 3.1 | 22.5 | 29.2 |
| 8 | ..... | 7.0 | 13.9 | 7.3 | ..... | 5.6 | 3.0 | 33.3 | 42.3 |
| 9 | ..... | 9.0 | 8.0 | 1.6 | ..... | 7.4 | 4.9 | 15.3 | 32.5 |
| 10 | ..... | 9.5 | 10.7 | 9.2 | ..... | 4.4 | 5.6 | 35.8 | 37.5 |
| 11 | ..... | 6.8 | 4.4 | .7 | ..... | 4.1 | 9.0 | 35.8 | 35.5 |
| 12 | ..... | 9.3 | 9.4 | 12.7 | ..... | 7.6 | 8.5 | 25.6 | 33.2 |
| Average * | ..... | **7.6** | **7.8** | **6.0** | ..... | **5.8** | **5.7** | **28.1** | **35.0** |

* The bold face figures are the arithmetical means of the values obtained in experiments 3 to 6 and 7 to 12, inclusive.

in the field of conjugated proteins. A third advantage lies in the possibility that integrated tissues may be amenable to division into unit complexes, each complex possessing an instability which is maximal at some definite $p_H$ value.

The faults of the method vary in accordance with the demands of subsequent manipulations designed to evaluate the effects of exposure of tissue to the various mediums. In the present studies the demands are not particularly rigorous, and the procedure fulfils the requirements.

*Losses of Weight by Cartilage During Extraction.*—Though there are several steps in the method for determining weight losses incidental to extraction, when duplicate samples of tissue weighing 50 mg. are carried through the procedure simultaneously, the difference between the final weights is less than 1 mg. The range of error appears to be greater when single determinations are made at different times on cartilage obtained from different sources. This is illustrated by the variation among values in each column of table 1. These figures show that the

maximum deviation of any value from the average of several values is about 5 per cent. This is principally attributable to errors incidental to weighing hygroscopic tissues at different times under conditions

TABLE 2.—*Percentages in Terms of Dextrose of the Samples Which, After Extraction, Were Converted by Hydrolysis into Substances Which Reduce Potassium Ferricyanide Under the General Conditions of Folin's Procedure* *

| Experiment | $p_H$ 4 | $p_H$ 5 | $p_H$ 6 | $p_H$ 7 1% NaCl | $p_H$ 7 10% $CaCl_2$ | $p_H$ 8.4 | $p_H$ 10 | $p_H$ 11 | $p_H$ 12 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | ..... | ..... | ..... | 22.60 | 14.14 | ..... | 15.59 | 7.60 | ..... |
| 2 | ..... | ..... | ..... | 22.34 | 19.75 | ..... | 19.75 | 15.28 | ..... |
| 3 | 29.60 | 26.10 | ..... | 28.00 | 20.22 | ..... | 18.95 | 7.87 | ..... |
| 4 | 28.50 | 30.50 | ..... | 25.10 | 26.50 | ..... | 30.40 | 16.10 | ..... |
| 5 | 26.70 | 27.50 | ..... | 31.90 | 27.70 | ..... | 27.90 | 15.00 | ..... |
| 6 | 29.70 | 23.50 | ..... | 32.00 | 28.80 | ..... | 26.40 | 18.70 | ..... |
| Average † | **28.62** | **26.90** | ..... | **29.25** | **25.81** | ..... | **25.91** | **14.42** | ..... |
| 7 | ..... | 32.00 | 32.10 | 32.00 | ..... | 26.00 | 31.60 | 22.80 | 13.10 |
| 8 | ..... | 35.30 | 31.90 | 33.90 | ..... | 38.50 | 34.50 | 21.20 | 11.70 |
| 9 | ..... | 29.90 | 28.00 | 28.70 | ..... | 29.50 | 29.10 | 19.10 | 7.90 |
| 10 | ..... | 27.20 | 30.80 | 29.80 | ..... | 30.70 | 29.50 | 10.40 | 9.10 |
| 11 | ..... | 28.80 | 29.30 | 28.50 | ..... | 31.60 | 27.90 | 8.50 | 7.00 |
| 12 | ..... | 26.90 | 28.90 | 31.20 | ..... | 29.40 | 28.30 | 14.30 | 9.60 |
| Average † | ..... | **30.01** | **30.17** | **30.68** | ..... | **30.95** | **30.15** | **16.05** | **9.73** |

* The samples of cartilage had been extracted with mediums having the hydrogen ion and salt concentrations designated at the heads of the columns; the tissues were then dried and weighed samples were hydrolyzed.

† The bold face figures are the arithmetical means of the values obtained in experiments 3 to 6 and 7 to 12, inclusive.

TABLE 3.—*Percentages of the Dry Weights of Cartilage Recovered as Sulfate from the Hydrolysates of Samples of the Tissue Which Had Been Extracted with Mediums of the Hydrogen Ion and Salt Concentrations Designated at the Heads of Columns*

| Experiment | $p_H$ 4 | $p_H$ 5 | $p_H$ 6 | $p_H$ 7 1% NaCl | $p_H$ 7 10% $CaCl_2$ | $p_H$ 8.4 | $p_H$ 10 | $p_H$ 11 | $p_H$ 12 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | ..... | ..... | ..... | 3.43 | 3.00 | ..... | 3.14 | 1.71 | ..... |
| 2 | ..... | ..... | ..... | 5.48 | 4.32 | ..... | 4.62 | 1.43 | ..... |
| 3 | 5.07 | 4.73 | ..... | 7.50 | 5.88 | ..... | 6.97 | 3.34 | ..... |
| 4 | 5.32 | 3.93 | ..... | 5.65 | 5.78 | ..... | 5.17 | 2.65 | ..... |
| 5 | 5.39 | 5.44 | ..... | 5.60 | 4.14 | ..... | 4.97 | 2.01 | ..... |
| 6 | 5.05 | 3.87 | ..... | 5.73 | 5.74 | ..... | 4.02 | 2.76 | ..... |
| Average * | **5.21** | **4.49** | ..... | **6.12** | **5.39** | ..... | **5.28** | **2.69** | ..... |
| Corrected average | **4.83** | **4.11** | ..... | **5.74** | **5.01** | ..... | **4.90** | **2.31** | ..... |
| 7 | ..... | 4.50 | 4.56 | 7.09 | ..... | 6.35 | 4.34 | 2.71 | 1.55 |
| 8 | ..... | 4.01 | 6.50 | 7.12 | ..... | 6.70 | 6.31 | 2.07 | 0.95 |
| 9 | ..... | 6.15 | 6.70 | 7.14 | ..... | 5.01 | 4.88 | 2.83 | .87 |
| 10 | ..... | 4.60 | 4.05 | 4.24 | ..... | 4.08 | 3.75 | 0.84 | .25 |
| 11 | ..... | 5.53 | 5.45 | 5.14 | ..... | 6.68 | 6.23 | 1.43 | .79 |
| 12 | ..... | 3.35 | 3.39 | 3.54 | ..... | 4.18 | 3.29 | .96 | .35 |
| Average * | ..... | **4.69** | **5.11** | **5.71** | ..... | **5.60** | **4.80** | **1.81** | **.79** |
| Corrected average | ..... | **4.46** | **4.88** | **5.48** | ..... | **5.37** | **4.57** | **1.58** | **.56** |

* The first line of bold face figures are the arithmetical means of the values obtained in experiments 3 to 6 and 7 to 12, inclusive, and the second line are the averages corrected for the average sulfate content of the reagents.

unfavorable to a standard state of dehydration. Despite the rather large deviations among the figures obtained in the several experiments, the average values shown in table 1 are probably a close approximation to

the actual weight losses of the average epiphysial cartilage during extraction in the various mediums.

The relationships among average weight losses in each series of experiments are shown in chart 1 and chart 2. The curves disclose that the weight stability of cartilage is a function of the hydrogen ion and the salt concentration of the mediums to which it is exposed. The point of maximum weight stability is at $p_H$ 10. With increasing acidity of the medium in the range $p_H$ 10 to 4, there is a progressive increment in loss of weight so that a point of minimum weight stability is reached at $p_H$ 4. With increasing alkalinity of the medium in the range $p_H$ 10 to 12, there is approximately a 30 per cent loss in weight, while in the range $p_H$ 10 to 4 the maximum weight loss is only about 4 to 5 per cent.

TABLE 4.—*Determinations on a Control Lot of Cartilage from One Source Used for Standardization of the Twelve Series of Determinations on Cartilages from Different Sources*

| Experiment | Reducing * Substances | Sulfate * |
|---|---|---|
| 1 | 22.60 | 3.43 |
| 2 | 22.60 | 4.15 |
| 3 | 21.81 | 3.40 |
| 4 | 21.10 | 3.28 |
| 5 | 23.50 | 3.18 |
| 6 | 21.70 | 3.91 |
| 7 | 23.90 | 3.13 |
| 8 | 22.50 | 3.20 |
| 9 | 23.60 | 4.00 |
| 10 | 22.30 | 4.06 |
| 11 | 23.60 | 3.23 |
| 12 | 24.10 | 4.18 |
| Average value | 22.78 | 3.60 |
| Maximum deviation from average value | 7.4% | 16.1% |

* Columns 2 and 3 are percentages of the dry weights of the control specimens recovered as reducing substances in terms of dextrose and sulfate from acid hydrolysates of the cartilage. The arithmetical means and the maximum per cent of deviations from these means are also tabulated.

In addition to the effect of the hydrogen ion concentration of the medium on weight loss, there is an influence which may be attributed to the concentration of salt in the medium. This influence is illustrated in chart 1, which depicts the relation between weight losses incidental to exposure of the tissue to neutral 10 per cent aqueous calcium chloride and losses accompanying treatment of duplicate lots of tissue with neutral 1 per cent aqueous sodium chloride. The cartilage is less stable in the concentrated salt solution, but the diminution in stability is so slight that there is no prominent break in the general form of the curve between the limits $p_H$ 10 and $p_H$ 4.

*Hydrolysis of Extracted Tissue.*—The third method used in this study degrades tissues to a state in which they are no longer suitable for morphologic studies. The principle of this particular method is acid hydrolysis. The objective is conversion of insoluble cartilaginous

residues into soluble components so that certain effects of extraction by buffer mediums may be investigated by methods designed for working with materials in solution.

Hydrochloric acid, rather than sulfuric acid, was used in conducting the hydrolysis because subsequent studies were concerned with the estimation of reducing substances and sulfate in hydrolysates. In our experience the conditions yielded hydrolysates with reproducible maximal values for reducing substances and sulfate. With this technic of hydrolysis, there were no important sources of error. The hydrolysates

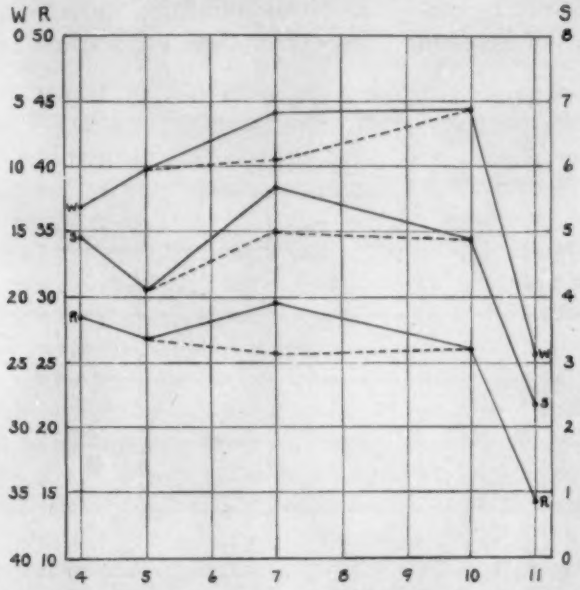


Chart 1.—This graph depicts in particular the relation between the effects on the weight, sulfate content and value for reducing substances when similar lots of cartilage are extracted at neutrality with 1 per cent aqueous sodium chloride and 10 per cent aqueous calcium chloride. The plotted values are the arithmetical means obtained by experiments 3 to 6, inclusive, and listed in tables 1 to 3. They include, in addition to data obtained at neutrality, values from similar lots of cartilage extracted at $p_H$ 4, 5, 10 and 11. The percentages of weight loss ($W$), reducing substances ($R$) and sulfate ($S$) are on the ordinate and the hydrogen ion concentrations of the extractive mediums are along the abscissa. The solid lines of the curves pass through values obtained after neutral extraction with the dilute salt solution, and the broken lines pass through values obtained after neutral extraction with the concentrated salt solution.

were clear, and though there was a tinge of color to the liquid, only a trace of dark brown residue remained on the paper after filtration.

*Reducing Substances in Hydrolysates of Extracted Tissues.*—The fourth method used in these studies is a means for the quantitative

determination in terms of dextrose of substances which reduce potassium ferricyanide under the general conditions of Folin's procedure. This method, as well as the preceding three methods, is suitable for studies at a microchemical level. So far as the present studies are concerned, 1 mg. of tissue is all that was required, though 5 to 6 mg. were ordinarily used.

The total procedure from the stage of fresh cartilage to the colorimetric determination has several advantages. It eliminates glycogen and dextrose, which are soluble in water. Each of these compounds would ordinarily be considered as uncontrollable variables because of the difficulty of preparing tissues with these components as constant

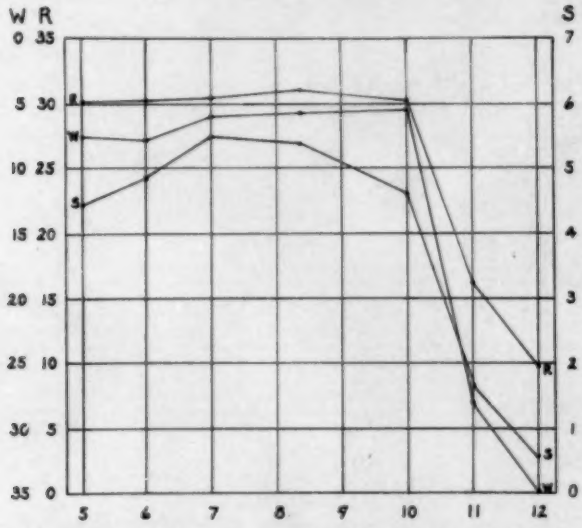


Chart 2.—This graph depicts the relation between the effects on the weight, sulfate content and value for reducing substances when similar lots of cartilage are extracted with mediums with different hydrogen ion concentrations, $p_H$ 5, 6, 7, 8.4, 10, 11 and 12. The plotted values are the arithmetical means obtained by experiments 7 to 12, inclusive, and listed in tables 1 to 3. The percentages of weight loss (*W*), reducing substances (*R*) and sulfate (*S*) are on the ordinate and the hydrogen ion concentrations of the extractive mediums are along the abscissa.

quantities. The method deals essentially, therefore, with reducing substances which are assumed to be degradation products of structural components of cartilaginous residues. The principal disadvantage of the method is that it is sensitive to diverse groups of reducing substances. A second disadvantage is the variable summation of errors involved in the manipulations required by the total procedure.

The results of the study of reducing substances are shown in table 2 and table 4. The figures represent the percentage by weight in terms of dextrose of reducing substances derived from cartilaginous residues by acid hydrolysis.

The data in table 4 were obtained by a study of one lot of tissue from which successive samples were taken as control material for standardization of the twelve separate experiments. The variations among these control determinations represent the range of error involved in all manipulations from the stage of final dehydration of extracted tissues to the completion of Folin's procedure. The maximum deviation of a single determination from the average value is 7.4 per cent of that value. This error plus the 5 per cent error which is the maximum deviation of any single determination of weight loss from the average weight loss in a series of successive determinations yields a total maximum deviation of about 12.4 per cent. This must be considered in the comparison of any single value with the average of several determinations.

The values shown in table 2 are experimental data obtained by studies of twelve lots of cartilage from 12 infants. Experiments 1 and 2 were not sufficiently comprehensive over the range of $p_H$ values, so that the data are not included in the averages. Experiments 3 to 6, inclusive, cover a broad range of $p_H$ values. They were designed particularly as a controlled investigation of the merits of 10 per cent aqueous calcium chloride as a means of modifying cartilage. Experiments 7 to 12, inclusive, extend over the same number of $p_H$ units as the preceding four experiments and fill certain gaps in the total range $p_H$ 4 to 12. The average values of the first set of four experiments and the second set of six experiments may be accepted as a first approximation to an accurate analysis of certain effects produced by the various buffer mediums. These average values are plotted as points on charts 1 and 2. Eleven single values shown in the fourth column of table 2 are plotted as points on chart 3.

The quantity of reducing substances seems to be influenced by several factors. The mediums which were used for the modification of fresh cartilage are of first importance. The preliminary washing of fresh sections with distilled water removes 0.6 to 0.02 per cent glycogen, if the data of Hoffmann and co-workers [3] are applicable. Furthermore, this treatment probably removes all dextrose and other diffusible, water-soluble reducing substances.

Not only is there a probable effect of water alone, but the salt content of water at neutrality has an influence. This is shown by chart 1, which depicts the relation between values for reducing substances in hydroly-

3. Hoffmann, A.; Lehmann, G., and Wertheimer, E.: Arch. f. d. ges. Physiol. **220**:183, 1928.

sates of cartilage extracted with 1 per cent aqueous sodium chloride and with 10 per cent aqueous calcium chloride. The difference in the average values is 3.44 per cent, and as the value obtained from cartilage treated with the concentrated salt solution is lower than that of the duplicate sample exposed to the dilute salt solution in five of six experiments, the effect can hardly be attributed to technical error. On the contrary, it is probably due to extraction of materials from the cartilage, as indicated by the forms of the curves representing sulfate values and weight losses.

The effects produced by a variation of salt concentration in the mediums are minor in comparison with those obtained by certain

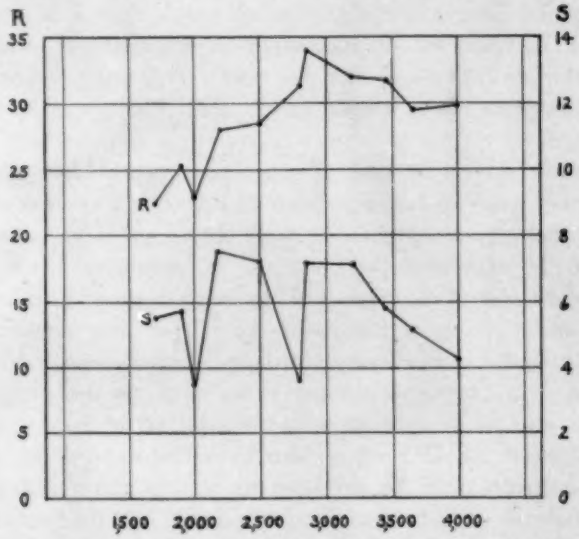


Chart 3.—This graph depicts the relation between the weight of an infant from whom a given cartilage was obtained and the corresponding values for reducing substances and sulfate derived from the cartilage by acid hydrolysis after extraction of the tissue with neutral 1 per cent aqueous sodium chloride. The values, which represent single determinations, are obtained from column 4 of tables 2 and 3. The percentages of reducing substances ($R$) and of sulfate ($S$) are on the ordinate. The weights of the infants from whom the cartilages were obtained are recorded in grams along the abscissa.

variations of the $p_H$ of the mediums. These effects are illustrated in charts 1 and 2. The general form of the curve for reducing substances is similar in each chart. The maximum value is at $p_H$ 8.4. Though a slightly lower value is obtained from tissues extracted at any other $p_H$ in the range $p_H$ 4 to 10, the form of the curve is almost linear with approximately equal positions of the end points of the curve at the extremes of the range. However, on the alkaline side of $p_H$ 10 there

is a sharp break in the stability of cartilage; the curve slopes sharply downward, passes at $p_H$ 11 through a low value representing a loss of about one half of the total quantity of reducing substances and terminates at $p_H$ 12 at a still lower value, which represents a loss of about two thirds of the total reducing substances. It seems probable in view of the forms of the curves which represent loss of weight and sulfate in the range $p_H$ 10 to 12 that the loss in reducing substances is due to degradation of tissue structure with conversion to a soluble form of the principal components which yield reducing substances on hydrolysis.

Another variable which seems to bear on the value of reducing substances is the stage of maturation of the cartilage in terms of ossification. In this series, ossification centers were present in femoral cartilages obtained from infants weighing 2,800 Gm. or more. The uppermost curve of chart 3 represents the coordinate relationships between values for reducing substances taken from column 4 of table 2 and the weights of 11 infants from whom the cartilages were obtained. This curve shows that the value of reducing substances increases with the weight of the infant up to about 2,800 Gm. This is the weight at which ossification centers, though small, are present. At this stage of maturation the values of reducing substances are maximal. They tend to decline slightly as the infants mature and as the ossification centers enlarge. Inasmuch as the cartilage of 1 infant, weighing 9,000 Gm., yielded a value approximating the average of the postossification values, plotted on the chart, it may be inferred that the slight decline is not continued as ossification centers undergo further enlargement.

*Sulfate in Hydrolysates of Extracted Tissue.*—The fifth method used in the study of cartilaginous residues is a procedure for the quantitative determination of sulfate ions. The method gives satisfactory values when the quantity of sulfate per cubic centimeter is 0.005 to 0.05 mg. It possesses maximum precision when the quantity of sulfate is about 0.025 mg. per cubic centimeter. Therefore, the method is theoretically suitable for the study of about 1 mg. of cartilage or one small frozen section.

Several substances interfere with the accuracy of the technic, but if the following precautions are taken, the method gives satisfactory results when applied to hydrolysates of cartilage. First, it is necessary to remove excess chloride ions. This was done by concentrating the acid hydrolysates in a hot salt water bath. Second, it is necessary to prove that the method gives near zero values on hydrolysates from which the sulfate has been precipitated with barium chloride. This was done in preliminary experiments, and zero or near zero values were obtained. Third, it is necessary to show that the barium precipitate is principally barium sulfate. This was done with several lots of cartilage and proof

obtained that the precipitate was never less than 99.5 per cent barium sulfate. Fourth, it is desirable to dilute hydrolysates so that the quantity of sulfate per cubic centimeter is approximately 0.02 to 0.03 mg. This was done whenever possible. Fifth, it is advantageous to run all controls and blanks as described in the section devoted to methods.

Even though these precautions were taken, it is evident from the data in table 4 that the maximum deviation among successive determinations on samples of a standard lot of dry control tissue is 16.1 per cent of the average of all determinations. This range of error is far from negligible but is tolerable because the maximum deviation from the average value amounts to only 0.0058 mg. sulfate per milligram of dry tissue. When simultaneous determinations are made on duplicate samples, the results are improved, the maximum deviation being about 5 per cent. The principal source of this error is in the judgment by color vision of the end point of the titration.

The results obtained in the twelve experiments are shown in table 3. The variations among single determinations and among the averages in each column of the two sets of experiments indicate that several factors influence the sulfate content. The range of error inherent in the technic is not the least of these factors. This is especially pertinent to an interpretation of the curve shown in chart 3 because this curve plotted from data in column 4 of table 3 connects points of single determinations in successive experiments rather than points of averages of several determinations. This curve discloses no systematic relationship between the sulfate content and either reducing substances or the stage of maturation of the cartilage. The values, on the other hand, seem to fluctuate at random above and below an arithmetical mean.

The use of distilled water in washing the sections of cartilage probably resulted in removal of all diffusible sulfate, so that this variable is eliminated and our studies thereby restricted to an estimation of sulfate which is bound in the tissue. The value of bound sulfate is influenced by the concentration of salt in the mediums to which the cartilage is exposed. This effect is illustrated by chart 1. This chart shows that there is less sulfate in cartilaginous residues extracted with 10 per cent aqueous calcium chloride than in duplicate residues extracted with 1 per cent aqueous sodium chloride. The difference in values is not great and might be considered as insignificant if there were not comparable reductions in the weight of the residues and the quantity of reducing substances. These coincidental data support the contention that a substance or substances yielding sulfate and reducing components on hydrolysis are removed from cartilage to a greater extent by the action of 10 per cent aqueous calcium chloride than by that of 1 per cent aqueous sodium chloride.

Although there is little doubt that the sulfate values are influenced at neutrality by the concentration of salt in the medium, much more pronounced effects on these values are obtained by certain variations of the $p_H$ of the medium. These effects are shown in charts 1 and 2 and are so represented that they can be compared with the effects of the dilute and concentrated neutral salt solutions. The values are at a maximum at $p_H$ 7 in the dilute salt solution. With increasing acidity, the values are reduced in the range $p_H$ 7 to 4, reaching a minimum at $p_H$ 5. With increasing alkalinity, there is a progressive reduction in the quantity of sulfate so that the value at $p_H$ 10 is about the same as that at $p_H$ 5. At $p_H$ 11 there is a sharp reduction in the value of sulfate. This loss is continued as the alkalinity of the medium increases, so that the quantity of sulfate in residues after extraction at $p_H$ 12 is zero or near zero.

The sulfate curves have four principal points of interest. These are at $p_H$ 5, 7, 10 and 12. At $p_H$ 5 the depressions of the curves which represent weight loss and reducing substances are relatively slight in proportion to the depression of the sulfate curve. A similar relationship exists between the three curves at $p_H$ 10, though here there is even less depression of the curves which represent reducing substances and weight loss. These data indicate that a fraction of the total bound sulfate is set free from the cartilage in the range $p_H$ 5 to 10 in quantities out of proportion to prediction. This fraction may be defined as labile bound sulfate. Apparently, it is retained in cartilage in maximum quantity at $p_H$ 7 in the presence of 1 per cent aqueous sodium chloride and in minimum quantity at $p_H$ 5 and $p_H$ 10. This fraction of bound sulfate, which is about 20 per cent of the total sulfate, is arbitrarily distinct from the residual principal fraction, which may be designated as stable bound sulfate. The distinction depends on the observation that the partial or complete loss of the principal fraction from cartilage is accompanied by a comparable loss of weight and reducing substances, while these values are not affected to a significant degree under conditions which lead to a loss of the labile fraction. The principal conditions under which the stable bound fraction is depleted are at neutrality in the presence of 10 per cent aqueous calcium chloride and in the range $p_H$ 10 to 12. In the neutral concentrated salt solution the depletion is slight but with increasing alkalinity in the range $p_H$ 10 to 12 the depletion is complete or nearly so. At $p_H$ 12 the average value for sulfate is 0.0056 mg. per milligram of dry extracted residue. In two experiments the value is very near zero, so that it is fair to assume that if the error incidental to incomplete washing of tissues after extraction at $p_H$ 12 is considered, all sulfate is lost by cartilage during exposure to a phosphate buffer solution at $p_H$ 12.

In view of the low value at $p_H$ 11 it seemed that prolongation of the period of extraction might result in complete removal of the residual sulfate. On the contrary, controlled experiments disclosed that no more

sulfate was lost by cartilage during seven days of extraction at $p_H$ 11 than was lost by duplicate samples during the standard twenty-four hour extraction period.

The reduction of sulfate to near zero values is accompanied by considerable loss of weight and of reducing substances, as indicated by the curves in charts 1 and 2. Hence the minimal conditions for complete extraction of sulfate are exposure of the prepared cartilage to the action of a phosphate buffer solution at $p_H$ 12 for twenty-four hours at 5 C., a loss of approximately 35 per cent in weight and a loss of about two thirds of the maximum quantity of reducing substances derived by acid hydrolysis of washed fresh cartilage. The forms of the curves indicate a close relationship among these simultaneous effects.

## INTERPRETATION OF DATA

Cartilage contains a polysaccharide which is known as chondroitin–sulfuric acid. There are two methods for extraction of this compound. The method which is in general use requires extraction of cartilage with strongly alkaline solutions, such as 2 per cent aqueous sodium hydroxide.[4] A more recent procedure requires extraction at neutrality with 10 per cent aqueous calcium chloride.[5] The steps in the isolation of the polysaccharide from the other extractives involve variable losses of the product. These losses increase in proportion to the care which is taken in the purification of the crude material. Though it is doubtful that a pure product has ever been obtained, the maximum yield of reasonably homogeneous material is about 20 per cent by weight of whole cartilage.

The location of the polysaccharide in cartilage and the means by which it is bound in the tissue are unknown. Because of the large quantity of polysaccharide, it is assumed that the compound must be present in the matrix rather than within the cells of cartilage. Furthermore, morphologists have demonstrated that the pericellular region of the matrix possesses distinctive staining affinities and have postulated that a relation exists between the polysaccharide and the staining affinities. Thus, it may be that the polysaccharide conforms topographically to the pericellular halo which is demonstrated by certain polychromatic basic dyes.

The spatial distribution of the compound in cartilage has not interested the chemist as much as has the type of chemical linkage responsible for holding the polysaccharide in the tissue. It is generally assumed

4. Levene, P. A. T.: Hexosamines, Their Derivatives and Mucins and Mucoids, Monograph 18, Rockefeller Institute for Medical Research, 1922.

5. Meyer, K.; Palmer, J. W., and Smythe, E. J.: J. Biol. Chem. **119**:501, 1937.

that the compound is bound to protein and this conjugated protein, though never isolated by acceptable methods, has been named "chondromucin." This hypothetic complex is unstable in the presence of strongly alkaline reagents, and hence the linkage by which the components of the complex are united has been characterized conservatively as "ester-like."[4] More recent studies cast doubt on this concept and provide data in accord with the idea that the polysaccharide is bound to tissue protein by acid-base salt linkages.[5]

The elementary composition of the polysaccharide and the properties of the forces by which it is bound in the tissue are of first importance in the interpretation of the results of the present studies. The compound is composed, theoretically, of tetrasaccharide units.[4] Each unit consists of two glycuronic acid and two galactosamine residues. The amino groups of the latter residues are acetylated, and the primary alcoholic groups are esterified with sulfuric acid. On complete acid hydrolysis, each unit yields galactosamine, glycuronic acid, acetic acid and sulfuric acid. Each of the derivatives galactosamine and glycuronic acid possesses one reducing group comparable with that of dextrose. Hence, the theoretic yield of reducing substances in terms of dextrose is about 75 per cent, and the yield of sulfate is about 20 per cent, of the total weight of the polysaccharide.

The preceding considerations form the basis for an interpretation of the present data within the limitations of two assumptions: first, that the polysaccharide contributes to the total sulfate a principal fraction defined as stable bound sulfate and, second, that the polysaccharide is the principal source of reducing substances derived from cartilaginous residues by acid hydrolysis. Reasons have been given for believing that these are conservative assumptions, and the following argument depends on their validity.

As a first approximation, it is desirable to convert the data with respect to weight loss, reducing substances and stable bound sulfate into polysaccharide units. It is convenient to consider the data in three parts: first, the results in the extraction interval $p_H$ 10 to 12; second, the results in the interval $p_H$ 4 to 10, and, third, the results obtained at neutrality with the dilute and concentrated salt solutions.

The key to the argument is the value for stable bound sulfate. In the extraction interval $p_H$ 10 to 12, stable bound sulfate has a maximal value at $p_H$ 10, and this is reduced to zero or near zero at $p_H$ 12. The maximal value coincides with the point of maximum weight stability of cartilage and with the point of near maximum value for reducing substances. This value for stable bound sulfate is about 4 per cent by weight of dry cartilage. This percentage when converted into polysaccharide units indicates that the quantity of polysaccharide in the average epiphysial cartilage is approximately 20 per cent. This is in accord with our

experience in chemical isolation of the polysaccharide. Our yields of purified material have varied from 15 to 18 per cent. At $p_H$ 12 the sulfate value is zero or near zero. Hence, the polysaccharide has been removed from the cartilage and the minimum possible loss of mass in the extraction interval $p_H$ 10 to 12 is 20 per cent of the weight of the cartilage at $p_H$ 10. But the reduction in weight is 30 per cent, and therefore it seems that simultaneously with removal of the polysaccharide the cartilage is deprived of another component or other components to the extent of 10 per cent of its total weight. In other words, when the polysaccharide is set free, it is accompanied by a small quantity of one or more substances.

Now, to what extent are these deductions substantiated by the values for reducing substances? If the average value for reducing substances is taken as about 30 per cent by weight of cartilage in terms of dextrose, the conversion of this value into polysaccharide units would lead to a value of about 40 per cent as the quantity of polysaccharide in cartilage. This is an excessively high value, so that it must be concluded that only a principal fraction of the total reducing substances is derived from the polysaccharide. This conclusion is substantiated by the data obtained in the extraction interval $p_H$ 10 to 12. In this interval the value for reducing substances decreases from about 30 per cent at $p_H$ 10 to nearly 10 per cent at $p_H$ 12. This loss of 20 per cent of the weight of cartilage as reducing substances in terms of dextrose is 5 per cent in excess of the theoretic loss attributable to the extraction of the polysaccharide. This means that either the estimation of the amount of polysaccharide is too low or that other components removed simultaneously with the polysaccharide contribute to the total value for reducing substances. The latter possibility seems the more probable because the cartilaginous residues retain about one third of the total quantity of reducing substances after removal of all polysaccharide.

The analysis of the data in the range $p_H$ 4 to 10 is difficult. The difficulty is attributable to a lack of correspondence between the curves for weight loss, reducing substances and sulfate. The divergence of the curves is at a maximum at $p_H$ 5 and $p_H$ 10. At these points the sulfate value is reduced to a significant degree without comparable reduction in values for reducing substances and weight. Hence the conclusion is reached that the extracted sulfate is labile and not bound to polysaccharide. It may be, however, that a small quantity of polysaccharide is removed in the extraction interval, especially at $p_H$ 5, where the weight loss is not negligible. This problem deserves further study because $p_H$ 5 is not beyond the physiologic range and because enzymatic action has not been excluded by these studies.

The third set of data subject to interpretation pertains to the action of dilute and concentrated salt solutions on cartilage at neutrality. The

curves in chart 1 show that there is a greater loss of weight, sulfate and reducing substances by cartilage extracted with 10 per cent aqueous calcium chloride than with 1 per cent aqueous sodium chloride. This occurred in five of six experiments and is attributed to removal of a small fraction of the polysaccharide. The effect is slight in terms of the total possible effect and is much less than that obtained by Meyer and co-workers, who reported essentially a complete extraction of polysaccharide at neutrality with 10 per cent aqueous calcium chloride.[5] The present experimental material and procedure, however, are different from those of the designated investigators.

If the data have been interpreted correctly, some deductions may be made concerning the forces which bind the polysaccharide in cartilage. A small fraction of the polysaccharide is apparently bound in the tissue by forces which are overcome at neutrality in the presence of a concentrated salt solution. According to theory, this is a dissociable fraction, and the linkages may best be interpreted as acid-base salt linkages, the acidic component being the polysaccharide and the basic component being tissue protein. We are not yet convinced that this "dissociable" fraction exists in fresh natural cartilage and believe that postmortem degradation must be excluded before proof is adequate. A large fraction of the polysaccharide is bound to the tissue by forces which are overcome within twenty-four hours at 5 C. in the presence of a phosphate buffer solution at $p_H$ 11. This is such a large fraction that it seemed but a matter of time to achieve complete extraction. On the contrary, prolongation of the period of extraction at $p_H$ 11 to seven days was no more effective than extraction for twenty-four hours. It became clear that the minimal conditions for overcoming all forces binding polysaccharide to cartilage are extraction at 5 C. for twenty-four hours with a phosphate buffer solution at $p_H$ 12. The data, therefore, support but one conclusion, namely, that the forces which bind the polysaccharide in the tissue are multiple and of unequal strength and that these forces vary among the polysaccharide units.

## SUMMARY

Fresh epiphysial cartilage obtained from infants was prepared in sections cut with a freezing microtome. The sections were washed in distilled water, divided into several equal lots, dried in vacuo and weighed. After rehydration in buffer mediums with concentrations ranging from $p_H$ 4 to 12 and in dilute and concentrated salt solutions at neutrality, the several lots of sections were exposed to the action of the designated mediums at 5 C. for twenty-four hours. At the completion of this period the sections were washed, dried and weighed so that the loss of weight incidental to the action of the mediums could be com-

puted. Subsequently, portions, usually 5 to 6 mg., of the extracted cartilaginous residues were converted into soluble components by acid hydrolysis. Finally, the quantities of reducing substances and sulfate in the hydrolysates were determined.

From averages of the numerical data it was concluded that chondroitin–sulphuric acid comprises about 20 per cent of the dry weight of the average epiphysial cartilage. Of this amount, a small fraction was extracted with neutral 10 per cent aqueous calcium chloride, and a large fraction was extracted at $p_H$ 11, while complete extraction in the range $p_H$ 4 to 12 was possible only at $p_H$ 12. These conclusions are an indication that the forces which bind the polysaccharide in cartilage are multiple and of unequal strength among the polysaccharide units.

The complete removal of this compound fails to account for the simultaneous removal over the range $p_H$ 7 to 12 of approximately 10 per cent of the total mass of cartilage, 20 per cent of the total reducing substances and 20 per cent of the total sulfate. Furthermore, the removal fails to account for the structural persistence of cartilaginous residues possessing 70 per cent of their neutral extraction weight, one third of the maximum quantity of reducing substances and little or no sulfate. These residues, as well as sections extracted elsewhere in the range $p_H$ 4 to 12, retain such form that they are suitable for study by morphologic methods which have as an objective the designation of the spatial distribution of certain components in integrated tissue structure.

# STUDIES OF CARTILAGE

## II. A QUANTITATIVE STUDY OF THE STABILIZING ACTION OF CRYSTAL VIOLET ON TISSUE POLYSACCHARIDE COMPOUNDS

GEORGE M. HASS, M.D.
NEW YORK

Previous studies have shown that chondroitin–sulfuric acid comprises about 20 per cent of an average ossifying human epiphysial cartilage.[1] A small fraction of this quantity was extracted with neutral 10 per cent aqueous calcium chloride. None was extracted at $p_H$ 10. A large fraction, approximating 60 per cent of the total quantity, was extracted at $p_H$ 11. Complete extraction of the polysaccharide in the range $p_H$ 4 to 12 was possible only at $p_H$ 12. These data pertain specifically to a character of chemical instability of one or more compounds composed of chondroitin–sulfuric acid and one or more other constituents of cartilage.

The present study is concerned with an attempt to modify this character in a reversible manner. The method of modification was devised on the assumption that a new order of instability might be acquired by the tissue polysaccharide compounds in the presence of an appropriate dye and that the original instability might be regained after proper degradation of the dye-tissue complex.

### METHODS

A description of the preparation of fresh frozen sections of epiphysial cartilage obtained from newborn infants has been given in a previous report.[1] Sections prepared in the designated manner were available for the present series of twelve experiments on cartilage from 12 infants.

The sections from each epiphysial cartilage, after repeated extractions at 5 C. with distilled water, were divided into three lots, dried in vacuo and weighed. Among the twelve experiments the weights of the several lots varied from 50 to 80 mg. In any one experiment the weights among the three lots varied no more than 5 mg.

After the initial dry weights had been obtained, the three lots of tissue were treated in the following way: One lot was placed in neutral 1 per cent aqueous sodium chloride, the second lot in a phosphate buffer solution at $p_H$ 11 and the third lot in distilled water. Within a few minutes all tissue was fully rehydrated and with a little agitation was dispersed as separate sections.

The sections which had been placed in distilled water were transferred to 200 cc. of a solution of crystal violet, the concentration of which was varied by

From the Department of Pathology, Cornell University Medical College.

1. Hass, G., and Garthwaite, B.: Arch. Path., this issue, p. 145.

choice from 3 to 12 mg. of dye per hundred cubic centimeters. The sections were stained for one hour, filtered from the solution over sintered glass disks, washed free from excess dye and transferred to a phosphate buffer solution at $p_H$ 11. The amount of dye remaining in the staining solution was estimated with a colorimeter. The three lots of tissue were extracted at 5 C. for twenty-four hours. After extraction at $p_H$ 7 and at $p_H$ 11, respectively, the two unstained lots of tissue were filtered over glass disks, washed and then dried in vacuo. The stained tissue, after extraction at $p_H$ 11, was handled in a different manner. During extraction these sections lost much of the original dark purple color. As the intensity of this color diminished, the medium acquired a light blue color which faded as a flocculent white precipitate settled to the bottom of the extraction flask. Care was taken to leave the precipitate undisturbed while the sections were transferred with forceps to a sintered glass funnel. Then the sections were decolorized with 95 per cent alcohol containing a trace of hydrochloric acid. The sections from which the dye had been extracted were subsequently washed with water and dried in vacuo.

For the determination of the amount of dye lost by the tissue during extraction at $p_H$ 11, the extraction fluid was neutralized with hydrochloric acid. During neutralization the white precipitate dissolved and the medium acquired a light purple color. This represented a quantity of dye which was estimated by comparison in a colorimeter with standard solutions prepared in a similar medium.

The amount of dye retained by the tissue during extraction at $p_H$ 11 was determined by colorimetric analysis of the quantity of dye in the diluted neutralized alcoholic extract. The standard solutions were prepared with concentrations of dye, alcohol and water similar to those of the unknown.

Three sets of quantitative colorimetric data were thus made available: first, the amount of dye absorbed by each milligram of tissue; second, the amount of dye set free from each milligram of tissue during extraction at $p_H$ 11, and, third, the amount of dye extracted with alcohol from each milligram of tissue after exposure of the stained sections to the action of a buffer solution, $p_H$ 11, at 5 C.

After desiccation in vacuo and at 50 C., the three lots of extracted tissue were weighed. The loss of weight incidental to extraction was thus obtained, and samples varying from 5 to 12 mg. were resected for hydrolysis. These were preserved until the completion of the final step in the first part of the experiment. This consisted of extraction of the residue of the decolorized tissue at $p_H$ 11 for twenty-four hours at 5 C. After this second extraction in an alkaline medium, these sections were filtered over a glass disk, washed, dried in a vacuum at 50 C., and weighed. A sample of tissue weighing 5 to 12 mg. was again resected from the dry mass of sections.

The four samples of extracted tissue and one sample of standard control lot of tissue were placed in separate pyrex tubes. Three cubic centimeters of four times normal hydrochloric acid was added to each tube and to one blank tube. The open end of each tube was sealed in a flame, and all tubes were heated at 100 C. for four hours. The hydrolysates thus obtained were handled as described in a previous report.[1] The amount of reducing substances was determined in terms of dextrose by the micromethod of Folin.[2] The amount of sulfate was determined by the micromethod of Cope.[3]

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2. Folin, O.: New England J. Med. **206**:727, 1932.
3. Cope, E. L.: Biochem. J. **25**:1183, 1931.

## RESULTS

*Quantities of Dye Absorbed at Neutrality and Retained at $p_H$ 11.*—The amount of dye absorbed by each milligram of tissue was directly proportional to the concentration of crystal violet in the staining solution and inversely proportional to the quantity of tissue. Among the twelve experiments the amount of dye absorbed by each milligram of tissue varied from 0.014 to 0.09 mg. The several values are shown in table 1. In this table the experiments are tabulated in an order depending on the quantity of dye absorbed. They are divided into three groups, from which average effects of low, medium and high concentrations of

TABLE 1.—*Data Pertaining to the Amounts of Crystal Violet in Cartilage and the Weight Stability of the Tissue at $p_H$ 11 Before and After Decolorization (see Chart 1)*

| | Dye, Percentage | | | Weight Loss, Percentage | | | |
|---|---|---|---|---|---|---|---|
| Experiment | Absorbed at $p_H$ 7 | Extracted at $p_H$ 11 | Extracted with Alcohol | $p_H$ 7 Tissue Unstained | $p_H$ 11 Tissue Stained | $p_H$ 11 Tissue Unstained | $p_H$ 11 Tissue Decolorized |
| 1 | 1.4 | 0.4 | 0.7 | 4.8 | 18.5 | 22.5 | 23.3 |
| 2 | 1.8 | 0.6 | 1.0 | 5.4 | 11.0 | 23.5 | 24.6 |
| 3 | 2.3 | 0.6 | 1.6 | 0.9 | 8.9 | 22.6 | 21.9 |
| 4 | 3.0 | 0.7 | 2.0 | 7.1 | 15.5 | 27.6 | 23.9 |
| 5 | 3.2 | 0.6 | 2.1 | 9.0 | 16.7 | 31.0 | 26.8 |
| Average | **2.3** | **0.6** | **1.5** | **5.4** | **14.1** | **25.4** | **24.1** |
| 6 | 3.6 | 0.5 | 2.7 | 10.0 | 11.3 | 23.7 | 22.0 |
| 7 | 4.1 | 0.7 | 3.1 | 4.6 | 3.3 | 23.4 | 21.7 |
| 8 | 4.8 | 0.6 | 3.6 | 7.2 | 9.4 | 25.3 | 26.8 |
| 9 | 6.0 | 0.8 | 4.9 | 5.7 | 7.1 | 25.7 | 24.2 |
| Average | **4.6** | **0.6** | **3.6** | **6.9** | **7.8** | **24.5** | **23.7** |
| 10 | 7.7 | 0.8 | 6.5 | 7.3 | 10.1 | 33.3 | 20.5 |
| 11 | 8.4 | 0.8 | 7.1 | 7.7 | 12.7 | 26.6 | 18.3 |
| 12 | 9.0 | 1.0 | 7.2 | 3.8 | 5.9 | 34.6 | 19.8 |
| Average | **8.4** | **0.9** | **6.9** | **6.3** | **9.6** | **31.5** | **19.5** |

the dye were calculated. The average quantity of dye per milligram of tissue was 0.023 mg. in the first group, 0.046 mg. in the second group and 0.084 mg. in the third group.

The second column of figures in table 1 shows the amount of dye set free from each milligram of stained tissue during extraction at $p_H$ 11. These values, which vary from 0.004 to 0.01 mg., depended slightly on the initial concentration of dye in the tissue, but to a greater extent on the hydrogen ion concentration of the extraction fluid. In this regard the observed effects at $p_H$ 11 may be described. As soon as the stained sections were placed in the buffer medium, the solution acquired a light purple color, and the intensity of color of the tissues was diminished. With the passage of time the solution gradually became colorless, a white precipitate settled to the bottom of the extraction flask and the tissues appeared to be almost completely decolorized. However, after com-

pletion of the period of extraction, it was possible to restore almost all of the original intensity of color of the tissue by placing the sections in a neutral buffer medium. Likewise, on neutralization of the alkaline

TABLE 2.—*Data Pertaining to the Amounts of Crystal Violet in Cartilage and the Amounts of Reducing Substances Derived from Dyed and Decolorized Tissue After Extraction at pH 11 (see Chart 2)*

| | Dye, Percentage | | | Reducing Substances, Percentage | | | |
|---|---|---|---|---|---|---|---|
| Experiment | Absorbed at pH 7 | Extracted at pH 11 | Extracted with Alcohol | pH 7 Tissue Unstained | pH 11 Tissue Stained | pH 11 Tissue Unstained | pH 11 Tissue Decolorized |
| 1 | 1.4 | 0.4 | 0.7 | 32.0 | 23.7 | 22.8 | 19.9 |
| 2 | 1.8 | 0.6 | 1.0 | 32.0 | 24.9 | 18.7 | 15.7 |
| 3 | 2.3 | 0.6 | 1.6 | 22.6 | 16.9 | 7.6 | 12.2 |
| 4 | 3.0 | 0.7 | 2.0 | 28.5 | 25.1 | 16.4 | 17.2 |
| 5 | 3.2 | 0.6 | 2.1 | 29.6 | 22.0 | 13.8 | 16.6 |
| Average | 2.3 | 0.6 | 1.5 | 28.9 | 22.5 | 15.9 | 16.3 |
| 6 | 3.6 | 0.5 | 2.7 | 22.3 | 23.0 | 15.3 | 16.2 |
| 7 | 4.1 | 0.7 | 3.1 | 28.6 | 28.0 | 13.8 | 15.6 |
| 8 | 4.8 | 0.6 | 3.6 | 28.7 | 26.8 | 19.3 | 18.6 |
| 9 | 6.0 | 0.8 | 4.9 | 31.9 | 31.2 | 15.0 | 18.9 |
| Average | 4.6 | 0.6 | 3.6 | 27.9 | 27.2 | 15.8 | 17.3 |
| 10 | 7.7 | 0.8 | 6.5 | 33.9 | 34.8 | 21.2 | 31.6 |
| 11 | 8.4 | 0.8 | 7.1 | 31.0 | 29.7 | 14.1 | 22.4 |
| 12 | 9.0 | 1.0 | 7.2 | 28.8 | 27.8 | 9.8 | 22.7 |
| Average | 8.4 | 0.9 | 6.9 | 31.2 | 31.1 | 15.0 | 25.6 |

TABLE 3.—*Data Pertaining to the Amounts of Crystal Violet in Cartilage and the Amounts of Sulfate Recovered from Dyed and Decolorized Tissue After Extraction at pH 11 (see Chart 3)*

| | Dye, Percentage | | | Sulfate, Percentage | | | |
|---|---|---|---|---|---|---|---|
| Experiment | Absorbed at pH 7 | Extracted at pH 11 | Extracted with Alcohol | pH 7 Tissue Unstained | pH 11 Tissue Stained | pH 11 Tissue Unstained | pH 11 Tissue Decolorized |
| 1 | 1.4 | 0.4 | 0.7 | 6.86 | 3.17 | 2.48 | 2.59 |
| 2 | 1.8 | 0.6 | 1.0 | 5.35 | 4.17 | 2.38 | 2.65 |
| 3 | 2.3 | 0.6 | 1.6 | 3.05 | 2.69 | 1.33 | 2.06 |
| 4 | 3.0 | 0.7 | 2.0 | 5.57 | 4.03 | 2.22 | 2.47 |
| 5 | 3.2 | 0.6 | 2.1 | 3.95 | 3.35 | 1.67 | 2.84 |
| Average | 2.3 | 0.6 | 1.5 | 4.96 | 3.48 | 2.02 | 2.52 |
| 6 | 3.6 | 0.5 | 2.7 | 5.10 | 4.96 | 1.05 | 1.67 |
| 7 | 4.1 | 0.7 | 3.1 | 5.92 | 5.59 | 2.56 | 2.25 |
| 8 | 4.8 | 0.6 | 3.6 | 5.11 | 4.72 | 2.60 | 2.80 |
| 9 | 6.0 | 0.8 | 4.9 | 5.22 | 5.38 | 1.63 | 1.93 |
| Average | 4.6 | 0.6 | 3.6 | 5.34 | 5.16 | 1.96 | 2.16 |
| 10 | 7.7 | 0.8 | 6.5 | 6.89 | 6.58 | 1.84 | 4.96 |
| 11 | 8.4 | 0.8 | 7.1 | 3.61 | 3.49 | 1.72 | 2.29 |
| 12 | 9.0 | 1.0 | 7.2 | 4.95 | 5.35 | 1.00 | 4.06 |
| Average | 8.4 | 0.9 | 6.9 | 5.15 | 5.14 | 1.72 | 3.77 |

extraction medium, the white precipitate promptly dissolved and imparted a light purple color to the solution. These findings indicated that the water-insoluble white color base of crystal violet was formed at $p_H$ 11 in solution and partly formed at $p_H$ 11 in the tissue. Therefore, the

amount of dye set free from the tissue during alkaline extraction was recovered and determined colorimetrically by conversion of the insoluble white color base to the ionized purple soluble salt form of the dye. In a similar way, any color base or other forms of the dye in the tissue, some of which were colored and others peculiarly crystalline, were regenerated and recovered for quantitative analysis by extraction of the tissue with alcohol containing a small amount of hydrochloric acid. The quantities are shown in column 3 of table 1.

Despite careful precautions, the quantity of dye initially absorbed by the tissue was never wholly recovered. The losses varied from 0.003 to 0.008 mg. per milligram of tissue. Although I cannot account for the losses, they were of such a constant range of magnitude that they did not seriously influence the evaluation of other colorimetric data.

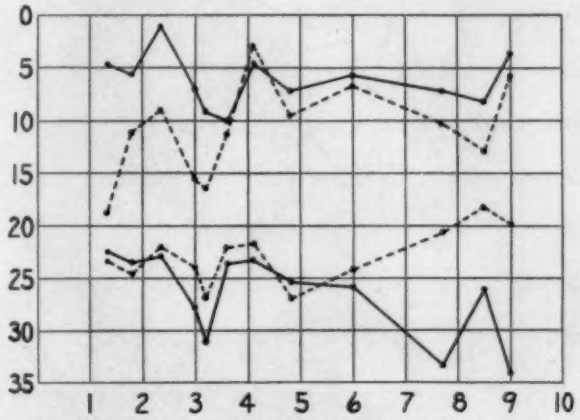



Chart 1.—The losses of weight by tissues during extraction as recorded in table 1 are plotted in per cent on the ordinate. The figures along the abscissa refer to per cent of crystal violet in tissues prior to extraction. Only one sample of tissue among the four samples in each of the twelve experiments contained dye, so that the amount of dye in this sample arbitrarily determined, for illustrative purposes, the abscissal position of the three unstained samples. The points are therefore distributed in twelve ordinate series of four.

The upper solid line passes through percental weight losses of control unstained tissue during extraction with 1 per cent aqueous sodium chloride.

The lower solid line passes through percental weight losses of control unstained tissue during extraction at $p_H$ 11.

The upper discontinuous line passes through the percental weight losses of stained tissue during extraction at $p_H$ 11.

The lower discontinuous line passes through percental weight losses of tissues which were stained with crystal violet, extracted at $p_H$ 11, decolorized with alcohol and then reextracted at $p_H$ 11.

The curves illustrate not only the quantitative relationships between the weight stability of tissues at $p_H$ 11 and the amount of dye in the tissues during extraction, but also the degree to which the stabilizing effects of the dye persisted after alcoholic decolorization of the tissues.

*Relations Between Amounts of Dye Retained by the Tissue and Maintenance of Tissue Weights, Reducing Substances and Sulfate at* $p_H$ *11.*—The losses of weight during extraction and the quantities of reducing substances and sulfate in hydrolysates of extracted tissues are shown in tables 1, 2 and 3. In each table the experiments are divided into three groups in accordance with the low, medium or high concentration of dye retained by tissue during extraction at $p_H$ 11. The data are arranged in four columns, and the average values obtained in each group of experiments are in bold face type.

The fourth and the sixth column of figures in each table show the values obtained by the three measurements on the two control lots of

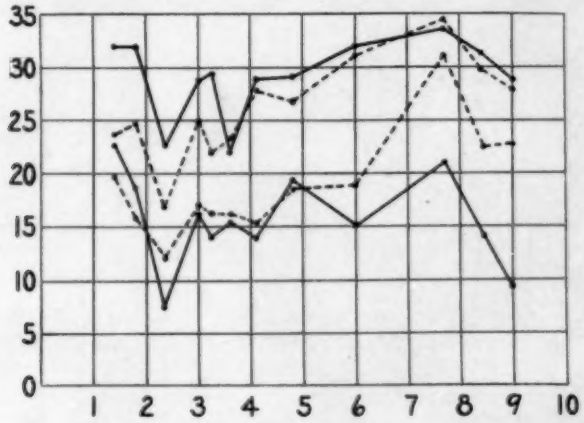



CHART 2.—The reducing substances recorded in table 2 are plotted in per cent on the ordinate of this graph. There were twelve experiments, and four lots of tissue were analyzed in each experiment. One of the four lots in each instance was stained with crystal violet. The amount of absorbed dye is recorded in per cent along the abscissa. This value also determines the position along the abscissa of the three other lots of tissue in each experiment, so that the values are plotted in twelve ordinate series of four.

The upper solid line connects reducing substance values of control unstained tissues extracted with 1 per cent aqueous sodium chloride prior to analysis.

The lower solid line passes through values for reducing substances of control unstained tissues extracted at $p_H$ 11 prior to analysis.

The upper discontinuous line connects values of reducing substances derived from tissues stained with crystal violet, extracted at $p_H$ 11 and then decolorized before analysis.

The lower discontinuous line passes through values for reducing substances derived from tissues which were stained with crystal violet, extracted at $p_H$ 11, decolorized with alcohol and then reextracted at $p_H$ 11 before analysis.

The curves illustrate not only the quantitative relations between the content of reducing substances derived from tissues after extraction at $p_H$ 11 and the content of dye in the tissues during extraction, but also the degree to which components yielding reducing substances resisted extraction after alcoholic decolorization of the tissues.

tissue in each experiment. The averages of these values in each corresponding group of experiments are similar to one another and to values obtained in previous studies, so that among the three groups there is no discrepancy due to random selection of cartilage.

Average values obtained by the three analyses of each control unstained lot of tissue were the standards by which the magnitude of the effects of staining the tissue before extraction and the reversibility of these effects by alcoholic decolorization prior to reextraction were determined. The figures in columns 5 and 7 of each table show that the effects of staining and decolorization were different in each group of experiments. These differences depended principally on the amount of dye absorbed by the tissue. When the tissue concentration of dye was

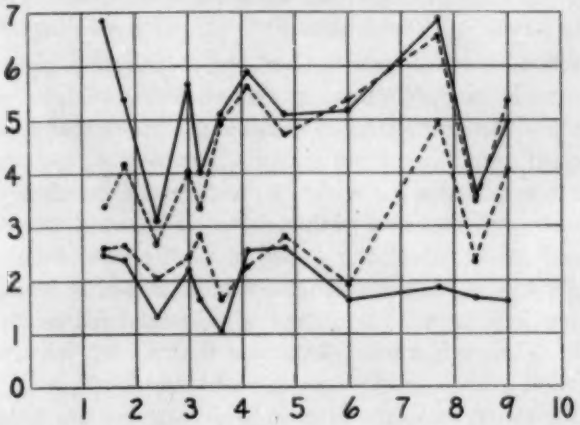



CHART 3.—The sulfate values recorded in table 3 are plotted in per cent on the ordinate of this graph. There were twelve experiments. In each experiment there were four lots of tissue for analysis. Of the four lots, one was stained with crystal violet. The amount of dye absorbed by this lot of tissue in each experiment is recorded in per cent along the abscissa. This value fixes the position along the abscissa of each of the three other lots of tissue, so that the forty-eight values are plotted in twelve ordinate series of four.

The upper solid line connects sulfate values of control unstained tissues extracted with neutral 1 per cent aqueous sodium chloride.

The lower solid line passes through sulfate values of control unstained tissues extracted at $p_H$ 11.

The upper discontinuous line connects sulfate values of tissues stained with crystal violet before extraction at $p_H$ 11.

The lower discontinuous line connects sulfate values of tissues which were stained with crystal violet, extracted at $p_H$ 11, decolorized with alcohol and reextracted at $p_H$ 11.

The curves show not only the coordinate relations between the contents of sulfate derived from tissues after extraction at $p_H$ 11 and the variable amounts of dye in the tissues during extraction but also the degree to which the capacity of dyed tissues to retain sulfate at $p_H$ 11 was modified by alcoholic decolorization of the tissues.

low during extraction at $p_H$ 11, as in experiments 1 to 5, the percental weight loss was less than that accompanying extraction of control unstained tissue at $p_H$ 11 but was greater than that recorded for unstained tissue extracted at neutrality. Corresponding relationships among values for reducing substances and sulfate were similar to those obtained by a study of loss of weight. When the concentration of dye in the tissue was medium or high, as in experiments 6 to 12, the tissue remained essentially stable during extraction at $p_H$ 11. The average value for each type of measurement of stability was very close to that of the unstained control tissue extracted at neutrality and quite different from that of unstained control tissue extracted at $p_H$ 11. The data show that stabilization of cartilage as defined by the three methods of measurement was complete at $p_H$ 11 when the concentration of dye in the tissue was greater than about 3.5 per cent.

*Relation Between Amounts of Dye Absorbed by Tissue and Maintenance of Tissue Weight, Reducing Substances and Sulfate at $p_H$ 11 After Decolorization.*—After the stained tissue had been extracted at $p_H$ 11, it was decolorized with alcohol and extracted again at $p_H$ 11. In this way tissue was made available for study of the reversibility of the stabilizing action of the dye. The results of this study are shown in the last column of each table. If the stabilizing action of the dye had been completely lost by decolorization, the figures in this column should be nearly identical with those obtained by a study of control unstained tissues after extraction at $p_H$ 11. There is a near identity of the two sets of figures in the first and second groups of experiments. In the third group of experiments, decolorization was not followed by a return of the cartilage to its usual unstable form. Partial stabilization persisted in rough proportion to the quantity of dye initially absorbed.

Charts 1, 2 and 3 depict the coordinate relationships among the data shown in the tables.

## COMMENT

The object of this study was to devise a histochemical method for reversible stabilization of a part of the integrated structure of cartilage. This structural part is chondroitin–sulfuric acid. Preliminary to formulation of a method it was necessary to determine conditions under which this component might be removed from cartilage and to select a dye which might be used for modifying these conditions.

It was found that a principal fraction of the polysaccharide was set free from the tissue during extraction with a phosphate buffer solution, $p_H$ 11, at 5 C. over a period of twenty-four hours.[1] This set of conditions was selected as suitable for the study of the stabilizing action of some dye. Among several possible choices, crystal violet was selected as the dye for four reasons: First, this dye is supposed to stain some

"mucins" in a characteristic metachromatic way. Second, this dye forms with tissues many highly colored complexes which are stable in water and unstable in alcohol. Third, the dye is converted into a water-insoluble white color base at the same hydrogen ion concentration at which the principal fraction of the polysaccharide is normally set free from cartilage. Fourth, the dye imparts such intense color to solutions that quantitative estimations can be made with a colorimeter at very low levels of concentration.

The essential plan of the method was twofold. First, a controlled study was made of the relation between concentrations of dye in cartilage and the amounts of polysaccharide persisting in the stained tissues during extraction at $p_H$ 11. Second, after removal of the dye from the extracted tissues with alcohol, they were again extracted at $p_H$ 11 and the amounts of polysaccharide persisting in the decolorized tissues were determined.

In following this plan it was necessary to study four similar lots of tissue in each experiment. One lot was extracted with neutral 1 per cent aqueous sodium chloride. This served as a control. A second lot of fresh sections was extracted at $p_H$ 11. This served as a second control. A third lot was stained with crystal violet prior to extraction at $p_H$ 11. After this treatment, the stained sections were decolorized with alcohol and again extracted at $p_H$ 11. Thus, except for intervening steps of decolorization and extraction, the fourth lot of tissue was identical with the third lot. In this way, all data concerning the stabilizing action of the dye and the reversibility of this action were essentially obtained by the study of one lot of tissue in each experiment.

The action of the dye was measured in three ways: First, the weight lost by extraction of each lot of tissue was determined. Samples of these extracted tissues were hydrolyzed, and the second and third determinations made on the hydrolysates. These determinations were concerned with the quantity of reducing substances and sulfate. If previous interpretations of the combined data obtained by these analyses are accepted, the principal fraction of losses of weight, reducing substances and sulfate at $p_H$ 11 is due to the release of chondroitin–sulfuric acid from the tissue. The minor fractional losses are due to a simultaneous liberation of additional unknown components.

With these comments in mind, the results given in tabular and graphic form lead to the following conclusions: First, the retention of chondroitin–sulfuric acid in cartilage was incomplete at $p_H$ 11 when the concentration of crystal violet in the tissue was less than about 3.5 per cent, but when the dye concentration was greater than 3.5 per cent, complete retention of the polysaccharide was the rule. Second, the capacity of stained tissue to retain polysaccharide at $p_H$ 11 was lost after decolorization with alcohol if the initial concentration of the dye in the

tissue did not exceed 6 per cent, but if the dye concentration exceeded this value, polysaccharide, which normally should be extractable, persisted in the tissue despite decolorization. Third, it was not possible to concentrate the polysaccharide in the tissue or to extract nonpolysaccharide compounds at $p_H$ 11 as a consequence of selective stabilization of tissue polysaccharide compounds by the dye.

The explanation of these results is obscure because the nature of the dye-tissue complexes which were formed is unknown. Crystal violet has an affinity for most structural components of cartilage, more for some than for others. This affinity while absorption is proceeding is directly proportional to the concentration of dye in solution and approaches zero when the concentration of dye in the tissue is 8 to 9 per cent. Once the dye-tissue complexes have formed, they exhibit a variable degree of stability in accordance with the composition of the medium to which the stained tissues are transferred. They are very stable in distilled water, fractionally stable in salt solutions of different ionic strength and totally unstable in alcohol.

If the affinity of the tissue for the dye is regarded as satisfied at the maximum dye concentrations of 8 to 9 per cent, it is clear that no more than a half reaction between dye and tissue was required for stabilization of tissue polysaccharide compounds at $p_H$ 11. Thus, if it is assumed that the dye was partitioned equally among the tissue components, though this was not actually true, one part of the dye by weight was required to stabilize six parts of the polysaccharide by weight. This represents an approximate proportion of one molecule of the basic dye to five sulfate radicals of the polysaccharide. When the dye-polysaccharide ratio was less than that necessary for complete stabilization, the polysaccharide was fractionally extracted from the tissue in proportion to the decrease in the ratio.

The means by which tissue polysaccharide compounds were stabilized in the presence of the dye is not known. Certainly, the bonds which hold the polysaccharide in the tissue were protected by the dye from the action of the alkaline solution. Otherwise, the polysaccharide would have gone into solution when the stained tissues after extraction at $p_H$ 11 were decolorized with alcohol and washed with water. Apparently, this protection persisted to some extent, despite decolorization, when a large amount of dye was initially bound in the tissue. It may be that this persistent action was due to a quantity of dye which resisted alcoholic extraction. If so, the quantity was very small and was never recovered on neutralization of the hydrolysates.

There would seem to be little doubt that the change in the dye from an ionized soluble salt to an insoluble white color base at $p_H$ 11 was an important factor in the mechanism of stabilization. This change was always complete in the solution in which the tissues were immersed. It

was never complete in the tissue. Though the tissues became pale at $p_H$ 11 and though the original intense stain reappeared on neutralization of the sections, no white deposits of the insoluble color base could be found in sections studied microscopically at $p_H$ 11. On the contrary, the only insoluble forms of the dye in the tissue were purple crystalline rosettes, which characteristically obscured each cell and lacunar space and which disappeared on neutralization of the tissue. The nature of these peculiar crystals remains to be studied.

Although this experimental method was suitable for reversible stabilization of tissue polysaccharide compounds, one desirable objective was not reached. The method failed as a means for maintaining polysaccharide in the tissue while other components were extracted. Hence, most components that were removed simultaneously with the polysaccharide at $p_H$ 11 from unstained tissues were stabilized to the same degree as the polysaccharide in the presence of crystal violet. This does not necessarily mean that the dye had no differential quality as a stabilizing agent. On the contrary, it probably means that the principal constituents which are extracted at $p_H$ 11 are related to one another in the fabric of tissue structure. Whatever the proper interpretation may be, recourse to the principles of this method may facilitate the isolation and chemical identification of some microscopic structures which thus far have been characterized only by their affinities for certain dyes.

## SUMMARY

When fresh frozen sections of infant epiphysial cartilage were extracted at $p_H$ 11 for twenty-four hours at 5 C., a large quantity of chondroitin–sulfuric acid and a small quantity of unknown tissue components were set free. When the sections were stained with crystal violet prior to extraction at $p_H$ 11, the polysaccharide became increasingly resistant to extraction in direct proportion to the concentration of dye in the tissue. This resistance was complete when the concentration of dye exceeded about 3.5 per cent. When the concentration of the dye did not exceed about 6 per cent, the stabilization of the polysaccharide in the tissue was reversible, because after extraction of the dye with alcohol the polysaccharide tissue compounds exhibited normal instability at $p_H$ 11. When the concentration of the dye was much greater than 6 per cent, stabilization of the polysaccharide was fractionally irreversible despite prolonged alcoholic decolorization.

By these methods of working with a stain which is supposed to possess certain elective affinities for compounds of chondroitin–sulfuric acid, it was not possible to maintain the polysaccharide in the tissue while extracting other components normally removed simultaneously with the polysaccharide at $p_H$ 11.

# STUDIES OF CARTILAGE

## III. A NEW HISTOCHEMICAL REACTION WITH HIGH SPECIFICITY FOR CARTILAGE CELLS

GEORGE M. HASS, M.D.
NEW YORK

This report is concerned with a new method by which a special property of the cytoplasm of cells may be studied. Though the exact nature of this property is obscure, the data which will be presented serve as partial proof that the property is not possessed by cells other than those of cartilage.

### MATERIALS AND METHODS

All tissues used in this study were obtained post mortem from infants and adults. Fresh blocks of tissue from the heart, lung, liver, kidney, adrenal, ovary, spinal cord, aorta, skin, bone, tendon, skeletal muscle and cartilage were available. Principal attention was directed to the selection of costal, patellar, semilunar, tibial, femoral, intervertebral and external aural cartilage from newborn premature and full term infants. A few studies were made of adult cartilage.

The fresh tissues, except when decalcification was necessary, were cut into sections with a freezing microtome set at 15 to 20 microns. The sections were divided into lots for preservation in water, alcohol and solution of formaldehyde U. S. P. The sections in distilled water were subdivided into three groups. One group was used for immediate study. Sections of the second group were mounted on glass slides and dried rapidly in a vacuum desiccator. The third group of sections was placed in the refrigerator at 5 C.

It was impossible to obtain suitable sections of fresh bone, although osseous tissue often remained so attached to epiphysial cartilages at the primary zones of ossification that a few observations could be made. Decalcification of formaldehyde-fixed or fresh tissue with half-molar hydrochloric acid was necessary before good sections of bone were obtained.

Sections of the designated organs and tissues were exposed to the action of numerous combinations and concentrations of half-molar hydrochloric acid, half-molar sodium hydroxide, fifth-molar sodium nitrite and four-hundredth-molar crystal violet. Optimum conditions for obtaining the desired reaction were as follows: A solution containing 10 cc. of half-molar hydrochloric acid, 4 cc. of four-hundredth-molar crystal violet and 1 cc. of fifth-molar sodium nitrite, or multiples thereof, was prepared. The reaction was allowed to proceed for forty-five minutes at room temperature. Then half-molar sodium hydroxide was added slowly until a delicate fluorescence developed. Seven to eight cubic centimeters was required, the quantity being so adjusted that a fine precipitate formed in the reaction mixture about ten minutes after appearance of the fluorescence. After addition of the alkali, 20 cc. of the reaction mixture was transferred to each of

From the Department of Pathology, Cornell University Medical College.

a desired number of 25 cc. beakers. One section of tissue was placed in each beaker and kept there throughout the fifteen to twenty minutes required for the formation of a precipitate. After precipitation was complete, ether was poured on the surface of the solution. The sections were withdrawn from the reaction mixture through the ether layer, washed in distilled water, floated onto glass slides, blotted with filter paper, stained with aqueous hematoxylin and mounted in glycerolgelatin under a cover slip.

Similar results were obtained when the sections had been affixed to glass slides by preparatory desiccation in vacuo. When available in this form, the tissues were more easily handled than when they were floating in the solution.

This standard method was varied so that some data might be gathered with respect to the action of alcohol, acetone, ether and half-molar hydrochloric acid on the capacity of the tissue to react and on the stability of the reaction product once it had formed. By use of these reagents it was possible to make a microscopic study of cells prior to running the reaction, after a positive reaction had been obtained and after eliminating all traces of the positive reaction. In other attempts to determine the nature of the reactive cytoplasmic component, the carmine stain for glycogen, the perosmic acid stain for lipids, the sudan IV stain for neutral glycerides and hematoxylin-eosin stains were prepared.

## RESULTS

*Morphology of the Tissue Reaction.*—A positive reaction consisted of an aggregate of particles in the cytoplasmic area of a cell. The particles, which varied from 1 to 3 microns in diameter, were yellow, refractile and discoid or spherical. They often had a biconcave form and always possessed curved margins.

The number of particles in the cytoplasmic area of cells was variable. When this variation was constant throughout a series of observations, the number of particles was accepted as an indicator of the reactivity of the cells. When no particles were found, it was assumed that the reactivity of the cells was zero. If fewer than ten particles were present in the cytoplasmic area, it was assumed that the cell had a low order of reactivity. Higher orders of reactivity were the rule in all preparations in which the cells gave a positive reaction. The maximum reactions were characterized by the presence of twenty-five to one hundred particles in relation to each cell. These reactions could be reduplicated if the technic was not varied, so that control tissues of a standard reactivity were always available for comparison with tissues of unknown reactivity. There was no explanation for the consistent variability in the reactivities of cells.

The distribution of particles was not at random. In poor preparations particles which were similar to those found in association with the cytoplasm of cells were often scattered over the surface of the tissue. In proper preparations this effect was absent. Here, there was an orderly pattern of particle distribution. They were alined in a single row or in a lattice of uniform spacing. The row of particles

usually formed a complete circle in one of two locations. The common location was just within the limit of the cytoplasmic membrane. In this position some of the particles occasionally appeared to be outside of the cell, but close scrutiny revealed invariable contact between these particles and the cytoplasmic membrane. A less common location of the particles was at the zone of transition between the axial cytoplasm, which in cartilage cells has an affinity for hematoxylin, and the reticular peripheral cytoplasm, which does not stain with hematoxylin. Though it was difficult to be certain in many instances, the conclusion was reached that the pale peripheral cytoplasmic area of the cell was chiefly concerned in the reaction, that the nucleus possessed no reactivity, that the particles were superimposed on rather than embedded in the cytoplasmic structure and that no cellular component distinguishable by the special staining methods was responsible for the reaction.

*Technical Variables Which Modified the Tissue Reaction.*—The reaction was very sensitive and during the initial studies seemed to be unusually capricious. However, reproducible results were obtained as soon as optimum conditions for running the reaction were found. In the search for these conditions it became clear that fresh sections were most suitable for study and that almost any radical treatment of the fresh sections modified the reaction. The least modification was produced by rapid desiccation of the fresh sections in a vacuum. A greater modification was produced by the action of a solution of formaldehyde on the tissue for a period of several hours. The capacity of the cells to react positively was lost if the tissues were in the solution of formaldehyde for several days, in half-molar hydrochloric acid for several hours, in distilled water at 5 C. for several days or in 95 per cent alcohol for a few seconds. Among the several modifying reagents and conditions, exposure of fresh moist sections to the action of alcohol proved to be most undesirable. The action of the alcohol was instantaneous in converting sections which normally gave a positive reaction into tissue which failed to give any reaction whatsoever. In view of these experiences, all controlled studies were conducted with fresh tissues preserved in the moist or dry state.

The degree of the reaction also varied in accordance with the absolute and relative concentrations of reagents in the reaction mixture. It was necessary that the acid-nitrite-dye solution have a hydrogen ion concentration greater than $p_H$ 3. Though it may be possible to replace hydrochloric acid by another strong acid, all efforts to obtain a positive reaction in acetic acid failed. Likewise, a certain degree of acidity was necessary during the interaction between the partially neutralized solution and the tissue. The best results were obtained at about $p_H$ 1 to 2, and no positive reaction occurred if the solution was alkaline.

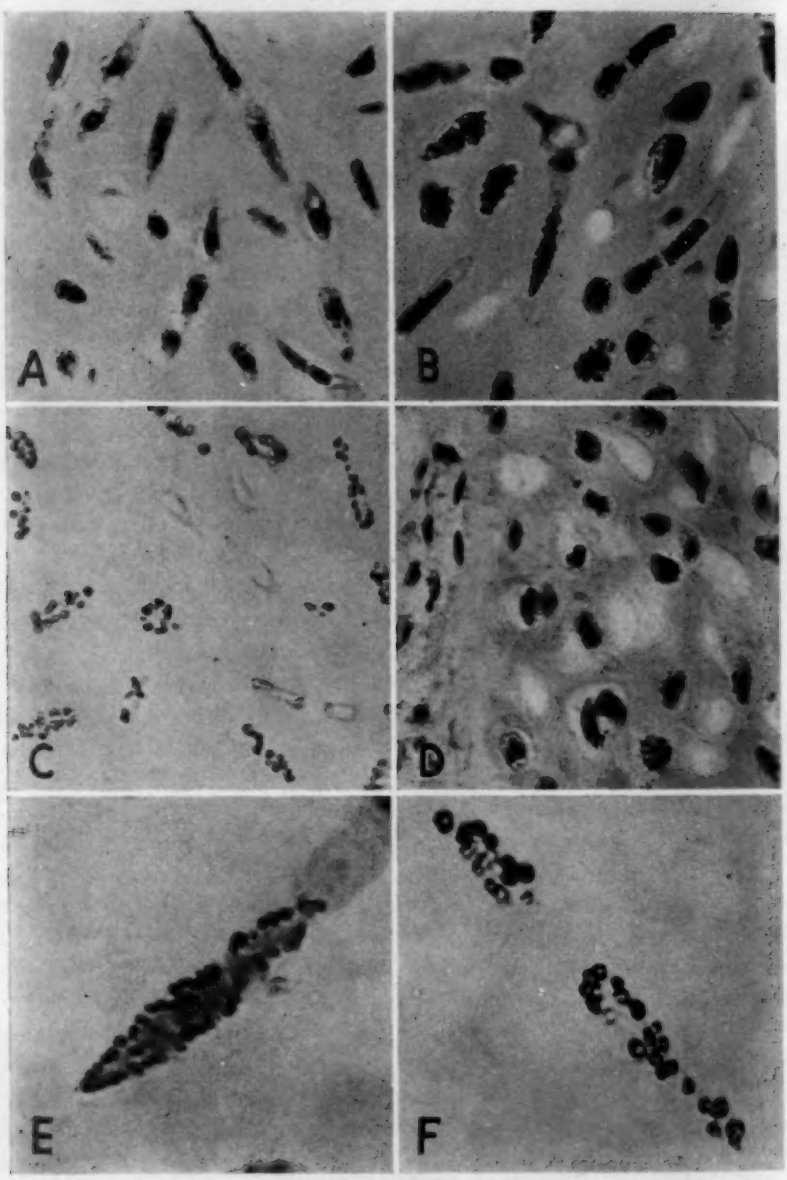
The concentration of dye in the reaction mixture influenced the results. At very low concentrations cells which normally gave a strong positive reaction exhibited a low order of reactivity or zero reactivity. With increasing concentrations there was a proportional improvement of the results.

Sodium nitrite was a necessary component of the reaction mixture. A trace of sodium nitrite was not sufficient. The amount prescribed in the directions for preparing the reaction mixture was near the lower limit of the necessary quantity. Larger amounts did not improve the results. The principal product derived from sodium nitrite under the experimental conditions was nitrous acid. It seems probable that the observed cytoplasmic reactions depended on modifications of the reactive cytoplasmic component, as well as of the dye, by this reagent.

The time variable was not a very important factor in either stage of the reaction. For instance, reaction mixtures often gave positive results even though the first stage of the reaction was varied between five and ninety minutes. The time necessary during the second stage depended principally on the rate of onset and progress of the precipitation. No reaction occurred prior to precipitation, so that staining effects in the ordinary sense were of no importance. When the precipitation was rapid, the reaction occurred with corresponding speed. The best results were obtained when the formation of the precipitate was slow and progressive over a period of fifteen minutes.

*Cytologic Specificity of the Tissue Reaction.*—Cells which gave a positive reaction were present in all types of infantile cartilage. Cells of adult cartilage did not react uniformly, so that additional studies of cartilage obtained from patients in various age groups will be made before definite conclusions are presented. Cells of noncartilaginous tissues did not give a positive reaction.

The cells of infantile epiphysial cartilage varied in their degree of reactivity in accordance with their stages of differentiation. The undifferentiated cells of the perichondrium and the neighboring fibrocytes of connective tissue did not react. The cells beneath the perichondrium gave a positive reaction which increased from low to high orders in rough proportion to the increment in the quantity of the intercellular matrix. Reactivity was possessed by all cells in the central part of the epiphysial cartilages but diminished in degree as the cells acquired the usual columnar alinement proximal to the line of ossification. Zero reactivity was exhibited by the large swollen cells proximal to the deposits of osteoid tissue. The few osteocytes which were distinguishable in fresh preparations at the osteochondral junction did not give a positive reaction. Other studies of osteocytes were unsatisfactory because the conditions which were used for decalcification destroyed the capacity of the control cartilage cells to react.




(See legend on opposite page)

As a rule, cells of costal and patellar cartilages gave the same type of reaction, though of a lower order, as cells at comparable stages of maturation and decay in tibial and femoral cartilage.

The cartilages of the intervertebral disks and the auricle of the ear were difficult to study because the fresh sections retracted, curled or distintegrated under the conditions of running the reaction. Despite the technical difficulties, some data were obtained. Cells of the hyaline cartilage of vertebral bodies gave a positive reaction. A similar reactivity was possessed by cells of the fibrocartilaginous peripheral part of the intervertebral disk and even by the widely dispersed stellate cells in the central part of the disk. The order of reactivity varied greatly among the numerous types of cells and tended toward negativity where pericellular collagenous fibers were abundant.

Cells of the elastic cartilage of the auricle of the ear gave positive reactions only when excellent preparations of the other cartilages were obtained with the same reaction mixture. These cells never possessed a high order of reactivity.

A low order of reactivity was also characteristic of most cells of the geniculate semilunar cartilages. Cells which gave a strongly posi-

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EXPLANATION OF FIGURE

These are photomicrographs of fresh infantile cartilage cut with a freezing microtome, subjected to the effects of the reaction mixture described in the text and mounted in glycerol-gelatin. The magnifications are as follows: *A*, *B*, *C* and *D*, × 400; *E*, × 1,000, and *F*, × 1,200.

*A* and *B* show cells of femoral epiphysial cartilage. The particulate reaction product is shown in the cytoplasm of cells in *B*, and its localization is brought out by staining the cell structure with hematoxylin.

Cells such as those in *B* acquired the appearance illustrated in *A* when the reaction product was removed with half-molar hydrochloric acid or alcohol. So far as structure was pertinent to this study, the cells in *A* were indistinguishable from cells which had not been treated with the reaction mixture.

*C* shows the positive cytoplasmic reaction in fresh unstained sections. Note the unreactive margins of the lacunar spaces from which the cells have fallen out during preparation of the sections.

*D* shows the perichondrium and subjacent matrix of costal cartilage. Note the absence of reactive particles in relation to cells of the perichondrium. Note the evidence of acquisition of the reactive cytoplasmic component as the cells become surrounded by the cartilaginous matrix.

*E* shows a single cell. The usual arrangement of the particulate reaction product in the pale peripheral cytoplasmic area near the cell membrane is emphasized here. Though the illusion of an affinity of the particles for hematoxylin is created, the particles are yellow by microscopic inspection.

*F* is a faithful reproduction of the particles when viewed in fresh sections. No special morphologic structure was found at the site of particles either before running the reaction or after removing the particles with several reagents.

tive reaction were surrounded by a hyaline matrix. Weaker reactions grading into negativity were encountered among the cells in the collagenous, fibrillar parts of the cartilage.

No detailed comments concerning the absence of reactions in noncartilaginous tissues are necessary. Suffice it to say that all types of cells, including osteocytes, osteoblasts and the cells occurring in mucinous tissues, have not been studied completely under appropriate conditions. Studies which have been made lead to the tentative conclusion that the reaction is highly specific for certain cells of cartilage.

## COMMENT

The present method, or modifications thereof, is a tool by which numerous morphologic problems may be studied. These studies will have to be conducted at an empiric level unless more data concerning the elementary factors which govern the reaction can be obtained. One factor seems to be a compound which is derived from crystal violet by the action of nitrous acid. A second essential factor seems to be some unknown cytoplasmic compound which is excited to react with the modified dye in the presence of nitrous acid. A third factor is the force by which the two components are bound together to give a positive reaction.

All data indicate that the first factor is a principal product of the action of nitrous acid on the dye. The properties of this product are such that it can be isolated from the reaction mixture by extraction with ether or by precipitation in particulate, pseudocrystalline form under conditions governing the reaction. Steps have been taken to purify this material.

The nature of the cytoplasmic compound necessary for the reaction is as obscure as that of the product derived from the dye. It is possible that it is a property rather than a definite compound. Whatever it may be, it is apparently possessed only by cells of cartilaginous tissues. Furthermore, these cells possess it to a variable degree in accordance with the type of cartilage in which they occur and their stages of differentiation. This cytoplasmic character is not identified with any microscopic structure or specific aggregates of structureless materials. It has a pattern of spatial distribution with the usual maximum reactive concentration in the region of the cytoplasmic membrane. It is not a compound or property of high stability, and its reactivity is modified or reduced to a low order by various reagents. Among these, alcohol is most effective. Immersion of this tissue in alcohol is sufficient to produce instantaneous inactivation. This does not necessarily prove that the reactive cytoplasmic component is soluble in alcohol, but the property does afford a convenient means for further study of the cellular factor which participates in the reaction. If the factor is a lipid,

it is not detectable by the usual staining reactions, nor is it present in noncartilaginous cells or in myelin in reactive form.

The forces which bind the modified dye to the cytoplasm are akin to those which are concerned in weak adsorptive reactions. For convenience of description, the modified dye has been regarded as particulate in form, though the particles adhering to the cell may possess a cytoplasmic component. Particles similar to those bound to the cytoplasm of cells are present in the reaction mixture. In this free form they have definite solubilities. The same solubilities are possessed by the particles associated with the cytoplasm of cells. Hence, if a true compound is formed by conjunction of the cytoplasmic component with the modified dye, it is either very unstable or it possesses solubilities similar to those of the uncombined particulate product derived from the dye.

In this regard, attempts to decompose and reform the particle–cytoplasmic compound may be mentioned. If half-molar hydrochloric acid is used to decompose the compound, the particles quickly disappear but the cytoplasm retains its reactivity, as proved by runing the reaction again on the same section of tissue. On the contrary, if alcohol or similar organic solvents are used to decompose the compound, the particles disappear and the cytoplasm loses its reactivity. Thus, one of these methods removes the particles and preserves the reactive cytoplasmic substance. The second procedure not only removes the particles but also either removes or inactivates the cytoplasmic material which enters into the reaction. So far as morphologic studies conducted by routine methods are concerned, the cellular structure remains unchanged, irrespective of the procedure used for removal of the particles. These facts limit the scope of further nonempiric studies of this reaction to the field of chemical analysis.

## SUMMARY

A new histochemical method for the study of the cytoplasm of cells has been described. This method consists of a microscopic interaction in the presence of nitrous acid between a particulate product derived from crystal violet and an unknown cytoplasmic component which is especially abundant in the region of the cytoplasmic membrane. Among numerous types of cells which have been studied, only those occurring in cartilage possess this component in reactive form. These cells differ in degree of reactivity in accordance with the type of cartilage in which they occur and their stages of differentiation. In infantile cartilage, which has been the principal source of material, the capacity to react is acquired by the cytoplasm as the cells differentiate from the perichondrium. It increases as the cells become embedded in a matrix but decreases to zero as the cells undergo decay at the zones of ossification.

# STUDIES ON INFLAMMATION

## IV. BEHAVIOR OF CELLULAR PROTEINASES IN EXPERIMENTAL TUBERCULOSIS OF RABBITS

CHARLES WEISS, M.D., PH.D.
SAN FRANCISCO

In recent years there has been an increasing tendency to study the mechanism of allergy and immunity in tuberculosis from the point of view of the phenomenon of inflammation. Allergy has been defined as "the capacity of the tissues of the infected animal to react with exaggerated and accelerated acute inflammation to reinfection." [1] During the first forty-eight hours of the allergic reaction to tuberculin there is an abundant accumulation of serum and polymorphonuclear leukocytes. These are later replaced by an infiltration of mononuclear phagocytic cells.[2] Acquired resistance to tuberculosis is likewise primarily a function of the cells. As the result of reinfection, the mononuclear phagocytes are mobilized with greater rapidity than on first infection. They possess an increased capacity for cell division and for engulfing and destroying tubercle bacilli.[3]

Since the classic work of Opie,[4] a generation ago, the proteolytic enzymes contained within leukocytes have been regarded as playing a dominant role in the chemical dynamics of the process of inflammation. Recent work from our laboratories [5] has extended these observations and has indicated that the three types of phagocytic cells—the polymorphonuclear leukocytes, the monocytes and the epithelioid cells which had been produced experimentally in the pleural cavity of rabbits by injections of aleuronat and starch, liquid petrolatum and a phosphatide derived from a strain of human tubercle bacilli, respectively—all con-

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From the Research Laboratories of the Mount Zion Hospital.

This investigation was aided by a grant from the Committee on Medical Research of the National Tuberculosis Association.

1. Lurie, M. B.: Immunology of Tuberculosis, in Piersol, G. M., and Bortz, E. L.: The Cyclopedia of Medicine, Surgery and Specialties, Philadelphia, F. A. Davis Company, 1939, p. 480.

2. Opie, E. L., cited by Menkin, V.: Dynamics of Inflammation, New York, The Macmillan Company, 1940.

3. Lurie, M. B.: J. Exper. Med. **69**:579, 1939.

4. Opie, E. L., and Barker, B. I.: J. Exper. Med. **10**:645, 1908; **11**:686, 1909. Opie, E. L.: Physiol. Rev. **2**:552, 1922.

5. Weiss, C.; Kaplan, A., and Larson, C. E.: J. Biol. Chem. **125**:247, 1938.

tained proteinases (cathepsins) with an optimum activity at $p_H$ 3.0, as well as dipeptidases which hydrolyze dl-alanylglycine at $p_H$ 8.0. The monocytes can be differentiated from the granulocytic leukocytes, since only the latter can split dl-alanylglycine at $p_H$ 5.5 and hydrolyze gelatin and casein at $p_H$ 8.0.

The present communication describes the behavior of cathepsins of various organs of rabbits which had been experimentally infected with virulent tubercle bacilli. Cathepsin was chosen as the first of the proteinases to be studied, since it has been recognized as of fundamental importance in the processes of cellular metabolism and growth.[6]

## METHODS AND MATERIALS

Four groups of white rabbits, each consisting of 5 to 8 animals, weighing 2,500 to 3,000 Gm., were prepared according to the agar focus technic developed by Lurie.[7] Group A were normal controls—each animal had received a subcutaneous injection of 5 cc. of sterile melted agar in the axillary region. Group B consisted of rabbits each of which received two or more successive intravenous injections of 1 mg. of a strain of tubercle bacilli of low virulence (R-1) at intervals of one month. This strain, according to Lurie, is more virulent than BCG and hence will give a greater acquired immunity. Thirty days after the last injection, when the R-1 bacilli had practically all died,[7] the animals were tested with serial dilutions of human and bovine tuberculin, 1:5, 1:100 and 1:1,000. Those which showed strongly positive reactions were then reinfected with a virulent bovine (Ravenel) strain of Mycobacterium tuberculosis by the agar focus technic. The rabbits were killed two weeks after reinfection and the tissues examined for content of cathepsin, as described in a later paragraph. Group C was prepared in the same manner as group B except that R-1 culture was used for reinfection of the allergic animals. The rabbits of group D were sensitized by repeated injection of the R-1 strain. After having become strongly positive in reaction to tuberculin, they were given subcutaneous injections of sterile agar.

*Preparation of Bacterial Suspensions.*—Homogeneous suspensions of tubercle bacilli, standardized so that 1 cc. contained approximately 1 mg. of bacteria, were prepared by emulsifying in a 0.5 per cent solution of sodium taurocholate (Difco).

*Extraction of Cellular Cathepsins.*—The rabbits were killed by exsanguination from the carotid artery, and the organs (liver, lung, spleen and kidneys) were preserved by rendering them lyophil in a Flosdorf-Mudd apparatus.[8] Within a period of about thirty days quantitative estimations of cathepsin were made on the frozen and pulverized tissues, according to the method of Anson[9]: 8 mg. samples were extracted with 0.5 cc. of water containing 1:50,000 merthiolate. Digestion of hemoglobin (Eimer and Amend) was carried out at 37 C. for thirty minutes at $p_H$ 3.0 to 3.5 as described in a previous communication.[5]

6. Goldshteyn, B., and Tsiperovich, A.: Biochem. J., Ukraine **11**:53, 1938; abstracted, Chem. Abstr. **32**:6679, 1938. Bromley, N. W., and Orechewitsch, W. N.: Biochem. Ztschr. **272**:324, 1934. Anson, M. L.: J. Gen. Physiol. **23**: 695, 1940.

7. Lurie, M. B.: J. Exper. Med. **63**:923, 1936.

8. Flosdorf, E. W., and Mudd, S.: J. Immunol. **29**:389, 1935.

9. Anson, M. L.: J. Gen. Physiol. **20**:565, 1937; **28**:79, 1938.

In experiments designed to test cathepsins derived from polymorphonuclear and monocytic leukocytes, exudates were prepared in the pleural cavities of rabbits with the aid of aleuronat-starch mixtures [10] or light liquid petrolatum, according to the technic of Lucké, Strumia, Mudd and McCutcheon.[11] After the cells had been washed three times with physiologic solution of sodium chloride, they were

*Cathepsin per Unit Weight of Tissue of Normal and of Reinfected Animals* *

| | Group | | | | Average Decrease in Cathepsin †§ |
|---|---|---|---|---|---|
| Spleens | 513 | 304 | 570 | 402 | |
| | 425 | 242 | 453 | 420 | |
| | 545 | 312 | 413 | 591 | |
| | 423 | 250 | 563 | 533 | |
| | 411 | 209 | 570 | 470 | |
| | 420 | 363 | | | |
| | | 246 | | | |
| Average | 456 | 290 | 514 | 483 | 193 |
| Livers | 127 | 94 | 83 | 119 | |
| | 225 | 83 | 141 | 216 | |
| | 248 | 89 | 141 | 272 | |
| | 216 | 76 | 138 | | |
| | 225 | 117 | 138 | | |
| | | | 146 | | |
| Average | 208 | 90 | 131 | 202 | 112 |
| Kidneys | 207 | 132 | 173 | 200 | |
| | 187 | 123 | 144 | 280 | |
| | 205 | 144 | 179 | 164 | |
| | 224 | 116 | 199 | 129 | |
| | 155 | | 161 | | |
| Average | 195 | 129 | 171 | 193 | 64 |
| Lungs | 169 | 97 | 68 | 134 | |
| | 134 | 128 | 153 | 63 | |
| | 146 | 119 | 165 | 166 | |
| | 156 | 94 | 139 | 168 | |
| | 173 | 107 | 143 | | |
| | 149 | | | | |
| Average | 154 | 109 | 133 | 133 | 24 |

* Each figure represents the average of duplicate determinations on an organ of an individual animal within the group. Catheptic activity is expressed as micrograms of tyrosine liberated from hemoglobin by enzyme extracted from 8 mg. of tissue.. Group A consists of normal rabbits given injections only of sterile agar; group B, allergic rabbits inoculated with virulent bovine (Ravenel) bacilli in an agar focus; group C, allergic rabbits inoculated with bacilli of low virulence (R-1) in an agar focus; group D, allergic rabbits given injections of sterile agar.

† Each figure represents the difference between the corresponding average values of group D and group B.

§ Mr. M. LeVita, statistician of the Fidelity Mutual Life Insurance Company of Philadelphia, has analyzed the data in this table by means of the "F test" (analysis of variance) and has found the conclusions to be justified.

centrifugated and ground in a mortar with sterile, washed sand and a small quantity of water and/or glycerin which had been previously adjusted with acetic acid to $p_H$ 4.0. The spleens, livers, kidneys and lungs were similarly extracted, either immediately after removal from the body or after having been rendered lyophil.

10. Weiss, C., and Czarnetzky, E. J.: Arch. Path. **20**:233, 1935.

11. Mudd, S.; Lucké, B., and Strumia, M.: J. Immunol. **29**:493, 1933.

### DEPRESSION OF CELLULAR CATHEPSINS IN ORGANS OF ANIMALS REINFECTED WITH TUBERCLE BACILLI (EXPERIMENTS IN VIVO)

As will be seen by reference to the table and the figure, the spleens of normal rabbits which had received sterile agar subcutaneously show concentrations of cathepsin per 8 mg. of ground, dehydrated tissue ranging from about 411 to 545 micrograms, measured as tyrosine (group A). When the animals were rendered allergic (positive reaction to tuberculin) by repeated inoculations with a strain of tubercle bacilli of low virulence, R-1, and were reinfected with the same microorganisms, there was no significant alteration in the concentrations of splenic cathepsin (group C). However, when a virulent bovine

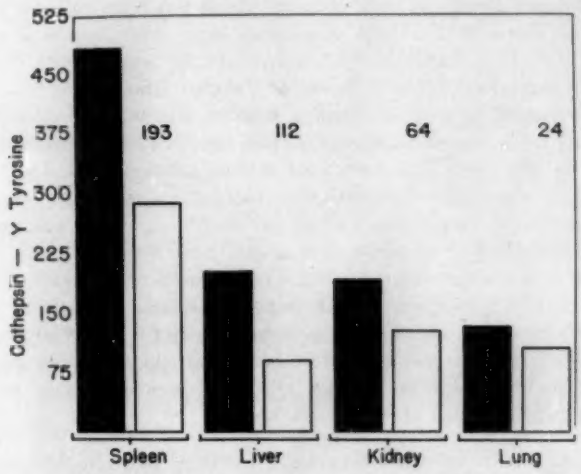


Chart showing distribution of cathepsin in normal and tuberculous spleens, livers, kidneys and lungs. The solid blocks represent normal organs (group A); the open blocks, organs of reinfected animals (group B).

(Ravenel) strain of M. tuberculosis was employed for reinfecting the animals, there was in most instances a marked decrease in the concentrations of splenic cathepsin—range, 242 to 363 micrograms (group B). Marked depressions were also observed in the livers of these rabbits. Of much less significance were the decreases in the kidneys, while the changes in the lungs were negligible.

It is of interest to note that the spleen and the liver, which show the largest decreases in concentration of cellular cathepsin, are in Lurie's experiments the organs which are the most efficient in destroying intravenously injected virulent tubercle bacilli on primary infection or on reinfection.[12] Furthermore, virulent bovine tubercle bacilli that leave

12. Lurie, M. B.: J. Exper. Med. **48**:155, 1928; **55**:31, 1932; **57**:181, 1933.

the site of reinfection from an agar focus are effectively destroyed on reaching the liver and the spleen, but less so when they reach the lung or the kidneys.[7]

### INHIBITORY ACTION OF TUBERCULOUS PHOSPHATIDE ON CELLULAR CATHEPSINS (EXPERIMENTS IN VITRO)

In an attempt to find an explanation for the aforementioned observations, a study was made in vitro of the effects of various chemical fractions obtained from human and bovine virulent tubercle bacilli—phosphatides, purified protein derivative, old tuberculin and a polysaccharide—on cellular cathepsins.

The proteinases of leukocytes and tissues were extracted from fresh material with the aid of water or 85 per cent glycerin which had been adjusted to $p_H$ 4.0 with tenth-nomal acetic acid. Each phosphatide was emulsified in a phosphate buffer solution, $p_H$ 3.1, to make final dilutions of 1:200 and 1:1,000. Tablets of purified protein derivative (Parke, Davis & Company, no. 2), each containing 0.05 mg., were crushed in a similar buffer solution and tested for inhibitory or accelerating activity in concentrations of 0.0083 mg. per cubic centimeter. The human and bovine old tuberculins were used in final dilutions of 1:150. Glycerin broth (supplied by the Cutter Laboratories) was set up in control experiments. A bovine polysaccharide (containing 0.2 mg. per milliliter) which was supplied by Dr. Michael Heidelberger of Columbia University, New York, was used in a final dilution of 1:40. The sources of the cellular cathepsins were normal polymorphonuclear leukocytes and monocytes, normal lungs and spleens and similar materials from rabbits which had been immunized with an R-1 strain and reinfected with a Ravenel culture of virulent bovine tubercle bacilli (group B). For purposes of comparison, tissues obtained from animals which had been immunized with strain R-1 but not reinfected (group D) were also tested.

Neither human nor bovine tuberculin, purified protein derivative nor the carbohydrate extracted from bovine tubercle bacilli exerted any effect on the leukocytic or tissue cathepsins of normal, allergic or reinfected rabbits in vitro. Only Anderson's phosphatide A-3, prepared from human or bovine virulent tubercle bacilli,[13] strongly inhibited the cathepsins of all the cells and organs tested.

### COMMENT

It has been shown in the present communication that the concentrations of cathepsins of the spleen and liver are markedly reduced in reinfected animals, i. e., in rabbits which had been immunized with a nonvirulent strain (R-1) and reinfected with a virulent bovine Ravenel culture of tubercle bacilli. These proteinases are not appreciably reduced in the lungs and the kidneys. Reinfection with nonvirulent cultures results in little or no decrease in tissue proteinase. In vitro experiments

13. Anderson, R. J.: J. Biol. Chem. **74**:537, 1927.

have demonstrated that the phosphatides of human and bovine tubercle bacilli markedly depress catheptic activity.

In view of the investigations of Lurie it is reasonable to interpret the results tentatively as follows: In the livers and spleens of reinfected animals which effectively destroy the tubercle bacilli that invade them from the agar focus, enough tuberculous phosphatide may be liberated from the disintegrated bacilli to inhibit catheptic activity. In the lungs and kidneys, however, where destruction of tubercle bacilli under these conditions is less pronounced, less phosphatide should be released and hence there should be less depression of catheptic enzymes. The fact that catheptic action is not affected by reinfection with the R-1 bacillus may be thought of as follows: It is well known that the R-1 bacillus causes no macroscopic tuberculosis beyond the site of inoculation in the highly susceptible, unvaccinated guinea pig. It may be safely assumed, therefore, that in the much more resistant, vaccinated rabbit, the R-1 bacilli did not leave the agar focus of reinfection at all, so that no bacilli invaded the livers and spleens of these rabbits. Therefore no phosphatide could be liberated in these organs and no depression of catheptic activity occurred.

The observed inhibitory activity of tuberculous phosphatide on leukocytic cathepsins and the absence of effect of the polysaccharide is in harmony with the findings of Wartman and Ingraham,[14] who showed that the former is toxic for human and rabbit leukocytes, reducing their motility and finally killing them, while the polysaccharide elicits only weak, negative chemotaxis.

The role of this inhibitory mechanism in the processes of caseation and softening is under investigation.

## SUMMARY

In rabbits, as a result of reinfection with a virulent strain of bovine tubercle bacilli, there is a marked decrease in the cathepsins of the spleen and liver. On the basis of in vitro experiments it is tentatively concluded that this may be due to the inhibitory action of the phosphatide which may be liberated during the destruction of the tubercle bacilli.

14. Wartman, W. B., and Ingraham, E. S.: Arch. Path. **29**:773, 1940.

# LEUKOCYTOSIS-PROMOTING FACTOR IN INFLAMMATORY EXUDATES OF MAN

VALY MENKIN, M.D.

M. A. KADISH, A.B.

AND

SHELDON C. SOMMERS, M.D.

BOSTON

The earlier studies of one of us [1] have demonstrated the presence of a leukocytosis-promoting factor in the inflammatory exudates of dogs and rabbits. Chemical investigation has indicated that this factor is a globulin or at least that it is apparently associated with the pseudoglobulin fraction of exudate.[2] The material presumably penetrates from the site of inflammation into the circulating blood, whence it is readily recovered.[3] The presence of the factor in the exudates of rabbits has recently been confirmed by Reifenstein and his collaborators.[4] The active factor has been found to be considerably more potent than a commercial pentnucleotide preparation; and it can likewise be obtained in a desiccated form without any great loss in its activity.[5]

These facts suggest the possible clinical application of this active material in various forms of infectious processes. It is well known that the prognosis of a number of such conditions is to a large extent referable to the number of circulating leukocytes.[6] It is therefore conceivable that the administration of the leukocytosis-promoting factor may yet prove to be clinically useful in some instances.

The present brief communication has been undertaken in an endeavor to determine whether the leukocytosis-promoting factor is present in the

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From the Department of Pathology, Harvard Medical School.

This is paper XXII of a series entitled "Studies on Inflammation."

This investigation was aided by grants from the Dazian Foundation for Medical Research, from the International Cancer Research Foundation and from the Jane Coffin Childs Memorial Fund for Medical Research.

1. Menkin, V.: (*a*) Am. J. Path. **16**:13, 1940; (*b*) Dynamics of Inflammation, New York, The Macmillan Company, 1940.

2. Menkin, V.: Arch. Path. **30**:363, 1940.

3. Menkin, V., and Kadish, M. A.: Arch. Path., this issue, p. 193.

4. Reifenstein, G. H.; Ferguson, J. H., and Weiskotten, H. G.: Am. J. Path. **17**:233, 1941.

5. Menkin, V.: Am. J. Path. **16**:685, 1940.

6. Robertson, D. H., and Fox, J. P.: J. Exper. Med. **69**:229, 1939. Fleisher, M. S.: Arch. Path. **30**:843, 1940.

inflammatory exudates of man. This, besides its obvious practical significance, would add further support to an understanding of the mechanism of leukocytosis in various clinical inflammatory conditions.

## EXPERIMENTAL PROCEDURE

All exudates were obtained from the pleural cavities of patients with empyema, which had generally resulted from some form of pneumonic involvement. In one instance, the exudate was derived from a patient with pulmonary tuberculosis.

The material was extracted for the presence of the leukocytosis-promoting factor essentially by the same technic employed for the purification of exudates from dogs.[3] The purified fraction was then injected, by cardiac puncture, into

TABLE 1.—*Effect of Leukocytosis-Promoting Factor Extracted from Human Exudate on the Number of Circulating Leukocytes of Dogs*

| Experiment | Absolute Number of Leukocytes Prior to Injection of Material (Per Cu. Mm.) | Highest Level of Leukocytes 13-28 Hr. After Injection of Material (Per Cu. Mm.) | Increase in Leukocyte Count (per Cent) |
|---|---|---|---|
| 1 | 16,350 | 35,700 | 118.3 |
| 2* | 17,050 | 22,200 | 30.2 |
| 3 | 15,800 | 45,600 | 188.6 |
| 4 | 20,300 | 40,300 | 98.5 |
| 5 | 14,200 | 27,250 | 91.9 |
| 6 | 12,750 | 34,750 | 173.6 |
| 7† | 22,450 | 33,550 | 49.4 |
| 8 | 21,200 | 41,050 | 93.6 |
| 9 | 13,700 | 35,950 | 162.4 |
| Average | 17,089 | 35,150 | 105.7 |

* No leukocytosis developed in the animal used in this experiment either with injection of any fraction containing the leukocytosis-promoting factor or with inflammation in the pleural cavity; nevertheless, this dog manifested with injection of the leukocytosis-promoting factor mentioned in the table a very marked "shift to the left" in the Arneth count, showing thus a discharge of immature granulocytes from the bone marrow into the circulation (table 2).

† Exudate from a patient with pulmonary tuberculosis was used in this experiment.

the circulating blood of essentially normal dogs. Total and differential leukocyte counts were performed approximately every hour for a period of about eight to fourteen hours. Several counts were also made during the subsequent two days. In the studies of the blood films, particular attention was paid to the percentage of immature granulocytes (i. e., forms with one lobe) by carefully making Arneth counts on 200 cells.

The results bearing on the absolute number of circulating leukocytes are shown in table 1. It is interesting to note that in practically every instance there was found a significant rise of the leukocyte level as a result of the injection of the human leukocytosis-promoting factor derived from about 30 to 50 cc. of the original material. The increase in the number of circulating leukocytes was considerably above that found as a result of normal variation.[7] The average rise of 105.7 per

7. Menkin (footnotes 1 and 2).

TABLE 2.—*Effect of the Leukocytosis-Promoting Factor from Human Exudates on the Blood Level of Immature Leukocytes*

| Experiment | Level Before Injection | | Approximate Time After Injection of Leukocytosis-Promoting Factor | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 Hour | | 3 Hours | | 6 Hours | | 12 Hours | | 24 Hours | | 48 Hours | |
| | One Lobe | Total * | One Lobe, per Cent | Total, per Cent * | One Lobe, per Cent | Total, per Cent * | One Lobe, per Cent | Total, per Cent * | One Lobe, per Cent | Total, per Cent * | One Lobe, per Cent | Total, per Cent * | One Lobe, per Cent | Total, per Cent * |
| Experiment 1 | 4.5 | 53.5 | | | | | | | 40 | 90 | | | | |
| Experiment 2 | 14 | 55.5 | 21 | 56 | 30 | 75 | 58 | 82.5 | 40 | 72.5 | 13 | 58.5 | 15.5 | 52.5 |
| Experiment 3 | 17.5 | 71.5 | 17.5 | 36.5 | 46 | 92.5 | 56 | 95.5 | 39.5 | 93.5 | 31.5 | 80 | 13 | 78.5 |
| Experiment 4 | 11 | 64.5 | 21.5 | 55.5 | 44 | 83.5 | 43.5 | 89.5 | 26.5 | 90 | 13.5 | 76.5 | | |
| Experiment 5 | 9 | 58 | 15 | 45 | | | 33 | 94 | 18.5 | 83.5 | 15 | 70 | | |
| Experiment 6 | 5.5 | 64 | | | 10.5 | 89.5 | 37.5 | 92 | | | | | | |
| Averages | 10.3 | 61.2 | 18.8 | 48.3 | 32.6 | 85.1 | 45.6 | 90.7 | 32.9 | 85.9 | 18.3 | 71.3 | 14.3 | 65.5 |

* The values refer to the total percentages of polymorphonuclear leukocytes.

cent surpassed even the effect induced by similar fractions from either dog or rabbit exudates.[7] Extraction of normal human serum yielded a fraction showing no detectable leukocytosis-promoting factor when

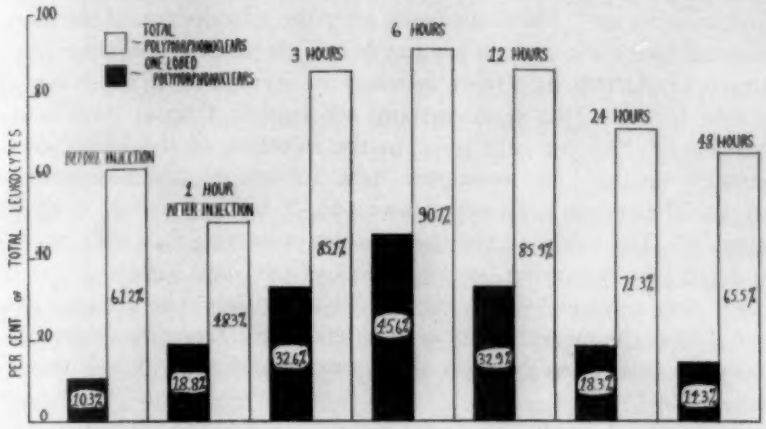


Chart 1.—The effect of the leukocytosis-promoting factor from human exudates on the discharge of leukocytes into the circulation (composite data of all experiments, table 2).

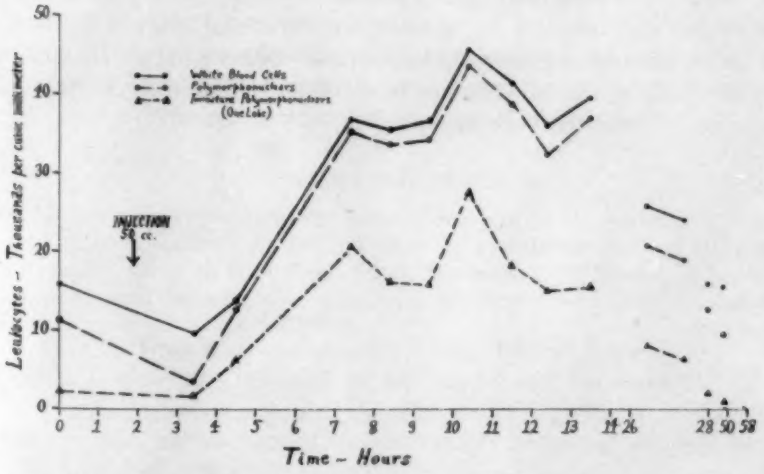


Chart 2.—The effect of the leukocytosis-promoting factor extracted from human exudate on the circulating white cells of a dog (experiment 3, tables 1 and 2).

injected into the blood stream of a dog. This is similar to the results obtained with normal dog serum.[8] The significant results derived from Arneth studies are compiled in table 2. For the sake of convenience only

8. Menkin (footnotes 1 *a* and 2).

the percentage of all polymorphonuclear leukocytes and that of immature granulocytes (one-lobe forms) are tabulated. It is readily seen that after the injection of human leukocytosis-promoting factor the total rise in the number of polymorphonuclear cells is correlated with a parallel increase in immature forms. About six hours after the introduction of the aforementioned factor the peak in the number of circulating immature forms is attained. At that time there is found an average of 45.6 per cent of one lobe forms. This is in striking contrast to a basal level in the circulation of 10.3 per cent prior to the injection of the leukocytosis-promoting factor. The composite data are graphically illustrated in chart 1. The course of an experiment (no. 3, tables 1 and 2) is shown in chart 2. The effect of the leukocytosis-promoting factor lasts, as a rule, for a period varying between one and two days (chart 2). It is perhaps also noteworthy and not wholly surprising that the exudative material from the pleural cavity of a patient with pulmonary tuberculosis seems to show a relatively lower concentration of leukocytosis-promoting factor (table 1).

## CONCLUSIONS

The leukocytosis-promoting factor found in inflammatory exudates of dogs and rabbits is likewise present in the exudative material of man. The leukocytosis-promoting factor is readily recovered as an active globulin fraction from various samples of empyema fluids. Its injection into the blood stream of a dog induces, within several hours, a significant rise in the number of circulating leukocytes. Differential counts indicate that the leukocytosis is doubtless referable to a discharge of immature granulocytes from the bone marrow.

# PRESENCE OF THE LEUKOCYTOSIS-PROMOTING FACTOR IN THE CIRCULATING BLOOD

VALY MENKIN, M.D.
AND
M. A. KADISH, A.B.
BOSTON

Previous studies by one of us [1] have indicated that in dogs and rabbits the mechanism of leukocytosis with inflammation is referable to the presence of a leukocytosis-promoting factor in inflammatory exudates. The presence of this factor in the exudates of rabbits has recently been confirmed by Reifenstein and his collaborators.[2] One of us [3] has succeeded in purifying exudates and recovering the active factor in the form of a pseudoglobulin or at least in a fraction that could not readily be dissociated from it. The earlier literature on the subject has been considered elsewhere [4] and therefore will be omitted here. In a previous study [1b] it was shown that neurogenic factors seem to be essentially ineffective in promoting the leukocytic reaction. The present brief communication presents data indicating the presence of the leukocytosis-promoting factor in the circulation of animals with acute inflammation. This fact suggests that the factor, probably by penetrating from the site of inflammation into the blood stream, ultimately induces by this route a discharge of granulocytes from the bone marrow.

## EXPERIMENTAL PROCEDURE

An inflammatory reaction was induced in a dog by an intrapleural injection of 1.5 cc. of turpentine.[5] On the following day a moderate degree of leukocytosis was usually found to have developed in this animal. A sample of blood was withdrawn from the heart. The quantities from different dogs varied from about 40

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1. Menkin, V.: (*a*) Am. J. Path. **16**:13, 1940; (*b*) Dynamics of Inflammation, New York, The Macmillan Company, 1940.

2. Reifenstein, G. H.; Ferguson, J. H., and Weiskotten, H. G.: Am. J. Path. **17**:233, 1941.

3. Menkin, V.: Arch. Path. **30**:363, 1940.

4. Menkin (footnotes 1 and 3).

5. Menkin, V.: Am. J. Path. **10**:193, 1934.

to 100 cc. In few animals did leukocytosis fail to develop as a result of the pleurisy induced. In such instances, the amount of leukocytosis-promoting factor present in the blood stream was found, as a rule, to be insignificant. The serum obtained was studied for the presence of the factor by modifying only slightly the chemical technic of extraction employed for exudates.[3] In brief, the material was treated with ammonium sulfate at one-half saturation. The precipitate mixture was dialyzed against running tap water for about one day. The indiffusible residual material was then reprecipitated with ammonium sulfate at half-saturation. The precipitate was separated from the remaining material by centrifugation and dialyzed against tap water until free of $SO_4^{=}$. The fraction remaining, which contained primarily the globulins of the serum, was injected by cardiac puncture into the circulating blood of an otherwise essentially normal dog.[4] The amount injected was usually calculated as the equivalent derived from approximately 15 to 25 cc. of the untreated

TABLE 1.—*Absence of the Leukocytosis-Promoting Factor from the Serum of Normal Dogs*

| Dog | Leukocyte Level Prior to Injection of Fraction Derived from Serum of a Normal Dog, per Cu. Mm. | Highest Leukocyte Level Attained After Injection of Fraction Derived from Serum of a Normal Dog, per Cu. Mm. | Change in Leukocyte Count, Percentage |
|---|---|---|---|
| 5-73 | 14,300 | 17,900 | +25.2 |
| 7-13 | 15,300 | 19,050 | +24.5 |
| 7-01 | 16,500 | 18,450 | +11.8 |
| 7-06 * | 15,300 | 17,000 | +11.1 |
| 7-13 | 11,900 | 13,150 | +10.5 |
| 7-08 † | 13,400 | 13,700 | + 2.2 |
| 7-08 † | 20,000 | 19,000 | — 5.0 |
| Average | 15,243 | 16,893 | +11.4 |

* Whole, unfractionated serum was utilized.

† No appreciable leukocytosis developed in this dog even when the animal had received an injection of the serum globulin fraction from a dog with inflammation. Nevertheless, under such circumstances the blood smears indicated a "shift to the left," i. e., a discharge of immature leukocytes.

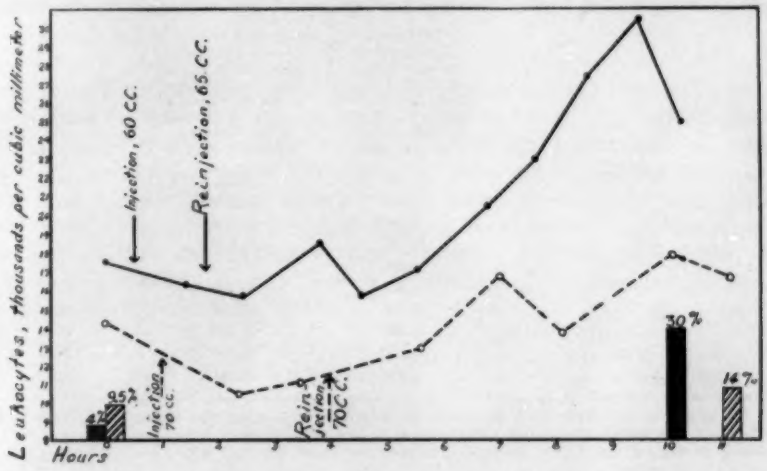
original serum. Total leukocyte counts were made approximately every hour; blood smears were studied in a number of instances in order to ascertain the differential forms of leukocytes.

Before injecting the inflammatory irritant into the thoracic cavity of each dog, a sample of blood was withdrawn from the heart, and the serum was extracted as described for the possible presence of any leukocytosis-promoting factor (LPF). A comparison of the effect on the number of circulating white cells obtained with the globulin fraction derived from the serum of a normal dog with that obtained with the globulin fraction derived from the same animal when there was concomitant acute pleural inflammation served as a rough estimate of the amount of leukocytosis-promoting factor in the blood stream.

The results of observations obtained with the serum globulin fractions from dogs prior to inducing an acute inflammation are conveniently assembled in table 1. It is clear that such material contains no appreciable

leukocytosis-promoting factor, the average increase in circulating white cells as a result of its injection being 11.4 per cent. These findings are in agreement with earlier observations.[4] A type experiment is graphically illustrated in the accompanying chart. A study of blood films indicates no significant discharge of immature leukocytes resulting from injection of such material (chart).

On the other hand, injection of the globulin fraction of serum taken from the same animals after induction of pleurisy reveals the presence of the leukocytosis-promoting factor in the circulating blood (table 2). The introduction of such a fraction into essentially normal dogs is followed by a significant increase in leukocyte counts, averaging 96.7 per cent. The induced leukocytosis, at least to a large extent, seems referable to a discharge into the circulation of immature granulocytes




The curves demonstrate the presence of the leukocytosis-promoting factor in the blood serum of an animal with inflammation. The solid line represents the leukocyte count with injection of the serum globulin fraction from a dog with inflammation; the broken line, that with injection of the serum globulin fraction from a normal dog.

At the foot of the chart, the solid black columns represent the immature leukocytes (one lobe) in the blood of the dog given an injection of the serum globulin fraction from a dog with inflammation, and the diagonally treated columns, the same for the dog given an injection of the serum globulin fraction from a normal dog.

(chart). A comparison of unfractionated samples of whole serum taken before and after inflammation likewise reveals the presence of the leukocytosis-promoting factor in the blood stream of animals with inflammation (dog 4 7-06, table 1, and dog 7-08, table 2).

The foregoing observations indicate therefore that the leukocytosis-promoting factor can be recovered from the circulating blood of an animal with an acute inflammation accompanied by leukocytosis. The presence of the active principle in the circulation offers a reasonable explanation for the effect of this agent on the bone marrow.[1a] It is conceivable in view of these observations that the findings of Longsworth, Shedlovsky and MacInnes [6] on the increase in alpha globulin in the blood serum of patients afflicted with various inflammatory processes may be referable to the penetration of the leukocytosis-promoting factor from the site of inflammation into the circulating blood stream. This

TABLE 2.—*Presence of the Leukocytosis-Promoting Factor in the Blood Serum of Dogs with Pleural Inflammation*

| Dog | Leukocyte Level Prior to Injection of Fraction Derived from Serum of a Dog with Pleural Inflammation, per Cu. Mm. | Highest Leukocyte Level Attained After Injection of Fraction Derived from Serum of a Dog with Pleural Inflammation, per Cu. Mm. | Increase in Leukocyte Count, Percentage |
|---|---|---|---|
| 7-04 | 11,100 | 38,550 | 247.3 |
| 7-06 | 10,900 | 31,950 | 193.1 |
| 7-03 | 9,000 | 21,100 | 134.4 |
| 5-73 | 17,500 | 30,450 | 74.0 |
| 7-13 | 13,000 | 21,850 | 68.1 |
| 7-08 * | 14,550 | 21,400 | 47.1 |
| 7-08 † | 11,900 | 17,150 | 44.1 |
| 7-08 * | 14,300 | 20,200 | 41.3 |
| 7-08 * | 18,850 | 23,750 | 20.6 |
| Average | 13,456 | 25,156 | 96.7 |

* No appreciable leukocytosis developed in this dog even when the animal had received an injection of the serum globulin fraction from a dog with inflammation. Nevertheless, under such circumstances the blood smears indicated a "shift to the left," i. e., a discharge of immature leukocytes.

† Whole, unfractionated serum was utilized.

interpretation has previously been suggested by one of us.[3] The experiments presented in the foregoing paragraphs add further support to this possibility, which is, however, now being investigated. The present observations coupled with earlier studies indicate that the leukocytosis-promoting factor is recovered from both the exudate and the serum of an animal with inflammation. Although the presence of the active material in the blood stream is presumably referable to its penetration from the site of inflammation, this still remains somewhat of an inference. Further studies are now in progress in an endeavor to establish

6. Longsworth, L. G.; Shedlovsky, T., and MacInnes, D. A.: J. Exper. Med. **70**:399, 1939.

this as a fact.[7] It has also been pointed out that the leukocytosis-promoting factor may prove to have definite clinical application in some infectious conditions.[8] Besides exudates as a source of the extract, the serum of animals with inflammation offers an additional possibility of obtaining the leukocytosis-promoting factor in substantial amounts for practical use.

## SUMMARY AND CONCLUSIONS

There is present in inflammatory exudates a globulin, termed the leukocytosis-promoting factor, which *per se* offers a reasonable explanation for the mechanism of leukocytosis with inflammation. The blood serum of a normal animal fails to reveal the presence of this substance. On the other hand, the leukocytosis-promoting factor is recovered in measurable quantities from the serum of a dog with an acute inflammatory reaction. The significance and possible application of this fact are pointed out.

7. An experiment was set up in which the exudate of a five hour inflammation was analyzed for its content of leukocytosis-promoting factor. This was compared with the amount of active material recovered from the blood serum after an inflammation of such brief duration. Whereas the leukocytosis-promoting factor was found present in the exudative material, it was completely absent in the sample of blood serum. These observations, in contrast to the findings in the blood samples from animals with inflammation of longer duration, definitely suggest that the leukocytosis-promoting factor is formed at the site of inflammation whence it penetrates into the circulating blood.

# EFFECT OF EXPERIMENTAL CIRRHOSIS ON THE INTRAHEPATIC CIRCULATION OF BLOOD IN THE INTACT ANIMAL

KHALIL G. WAKIM, M.D.*
AND
FRANK C. MANN, M.D.
ROCHESTER, MINN.

Ever since the early days of Vesalius, who is supposed to have been the first to describe cirrhosis of the liver, literature has been accumulating on various kinds of cirrhosis and their etiologic agents. Many of the investigators have shown that repeated inhalation of carbon tetrachloride is an efficient procedure for the production of experimental cirrhosis. This study was undertaken in an attempt to determine the various changes that take place in the intrahepatic vascular systems of living animals during the production of experimental cirrhosis by the inhalation of carbon tetrachloride.

Kretz [1] found in cirrhotic livers islets of hepatic cells from which several tributaries of a single branch of the hepatic vein drained the blood. In these islets the hepatic tributaries were located eccentrically. These vessels he considered to be the central veins brought to their eccentric positions by asymmetric degeneration and regeneration of hepatic cells. Injections through the portal vein left the islets very slightly injected or completely unpermeated, but injection through the hepatic artery completely permeated them. Gardner and his associates [2] gave carbon tetrachloride to various animals through different routes and detected necrosis in the liver in twelve hours. The lesions became maximal in forty-eight hours. These authors came to the conclusion that carbon tetrachloride causes central necrosis of the liver, no matter by what route it is administered. Lamson and Wing [3] studied the effect of repeated doses of carbon tetrachloride in the dog over long periods. They reported cirrhosis of the liver with necrosis, inflammatory reaction and atrophy, followed by hypertrophy and scarring. One of us (Mann)

* Fellow in physiology at the Mayo Clinic.

From the Division of Experimental Medicine, Mayo Foundation.

1. Kretz, R.: Internat. Clin. **3**:289, 1905.

2. Gardner, G. H.; Grove, R. C.; Gustafson, R. K.; Maire, E. D.; Thompson, M. J.; Wells, H. S., and Lamson, P. D.: Bull. Johns Hopkins Hosp. **36**: 107, 1925.

3. Lamson, P. D., and Wing, R.: J. Pharmacol. & Exper. Therap. **28**:399, 1926; **29**:191, 1926.

and associates [4] gave carbon tetrachloride to dogs every two to five days for periods extending from four to eight months and produced cirrhosis. Their findings are in agreement with those of Lamson and Wing. They noted that in experimentally produced cirrhosis the nodules of hepatic parenchymatous cells were dissociated from, and unrelated to, the blood vessels and biliary ducts of the liver. The arrangement of the vascular channels within the injured lobules was abnormal, and this limited the regenerative capacity of the injured hepatic tissue.

In his study of the vascular lesions in cirrhotic livers, McIndoe [5] observed a marked diminution in the total intrahepatic vascular bed. No normal central veins were found, and the regenerated lobules received blood chiefly from the branches of the hepatic artery. He stated that in portal cirrhosis the hepatic parenchymatous cells are divorced from their normal portal blood supply and in the long run become nourished almost entirely by the hepatic artery. Lacquet [6] found that in adult rats carbon tetrachloride caused fatty degeneration with central necrosis in twenty-four hours but that complete regeneration took place in two weeks. If the injections of carbon tetrachloride were repeated, cirrhosis of the liver developed. Moon [7] reported that carbon tetrachloride is one of the agents which produce a condition that meets the rigid criteria for Laennec's cirrhosis. He concluded that cirrhosis is chronic diffuse inflammation of the liver. Cameron and Karunaratne [8] administered carbon tetrachloride to rats orally or subcutaneously and obtained marked degeneration and necrosis of the lobules, followed immediately by proliferation of the unaffected hepatic cells.

Higgins and Stasney [9] produced atrophic cirrhosis in albino rats by brief daily exposures to the fumes of carbon tetrachloride. Severe macrocytic anemia was noted, and the hepatic parenchyma was markedly destroyed. There were enormous hyperplasia of the periportal connective tissue and marked proliferation of the biliary tubules. White [10] studied the effect of single and repeated subcutaneous injections of carbon tetrachloride in young pigs. A single injection caused necrosis of the central half of the lobule, but repair was complete in one week. Repeated injections produced cirrhosis.

4. Mann, F. C.; Fishback, F. C.; Gay, J. G., and Green, G. F.: Arch. Path. **12**:787, 1931. Bollman, J. L., and Mann, F. C.: Ann. Int. Med. **5**:699, 1931. Mann, F. C., and Bollman, J. L.: Tr. A. Am. Physicians **53**:145, 1938.

5. McIndoe, A. H.: Arch. Path. **5**:23, 1928.

6. Lacquet, A. M.: Arch. Path. **14**:164, 1932.

7. Moon, V. H.: Arch. Path. **13**:691, 1932; **18**:381, 1934.

8. Cameron, G. R., and Karunaratne, W. A. E.: J. Path. & Bact. **42**:1, 1936.

9. Higgins, G. M., and Stasney, J.: Folia haemat. **54**:129, 1936. Stasney, J., and Higgins, G. M.: ibid. **55**:93, 1936.

10. White, E. G.: J. Path. & Bact. **49**:95, 1939.

## METHODS

In order to study the effects of carbon tetrachloride on the intrahepatic circulation in albino rats the inhalation method of administration was used. The rats were placed in a specially constructed chamber in which the circulating air was saturated with carbon tetrachloride vapors. A large number of young rats were used in two series, each series consisting of several groups. To one series the carbon tetrachloride was administered only once for a period of thirty minutes. To the other series the carbon tetrachloride was administered daily for a period of ten to thirty minutes, depending on the size and age of the rats. These daily exposures were carried on for about three months, during which time cirrhosis of the liver developed. Before as well as after the administration of the carbon tetrachloride the rats were kept in their cages, in which they received ordinary feed and water ad libitum. Another group of rats was given carbon tetrachloride subcutaneously in single doses amounting to 0.1 cc. per hundred grams of body weight.

Immediately after a group of rats was exposed to the carbon tetrachloride vapors, a few were anesthetized with ethyl carbamate (urethane), pentobarbital sodium or ether, and their livers were exposed for study of the intrahepatic circulation in situ. Other rats from such a group were anesthetized and their livers studied at intervals ranging from one hour to several weeks after their single exposure to carbon tetrachloride. The observations on the intrahepatic circulation were made by the quartz rod transillumination technic, in rats maintained at a constant body temperature.

## RESULTS

*Effects of a Single Exposure to Carbon Tetrachloride Vapors.*—With scarcely any exception, the macroscopic appearance of the livers immediately after the half-hour inhalation of carbon tetrachloride revealed slight congestion, while microscopic examination under low and high powers showed a moderate increase in circulatory activity during the first two to three hours after inhalation of carbon tetrachloride. By placing a swab saturated with carbon tetrachloride to the nose of the rat during the microscopic observation one could see transient intrahepatic vasoconstriction with paling of the liver while the rat was inhaling the carbon tetrachloride, but within half a minute after the swab was taken away from the nose, the original condition of the circulation returned. Similar vasoconstrictor reactions to brief inhalations of carbon tetrachloride vapors were manifested by the livers of control animals which had not been exposed previously to carbon tetrachloride.

Within four to eight hours after the half-hour exposure to carbon tetrachloride, macroscopically the livers appeared pale and dirty gray with minute reddish regions scattered irregularly throughout the hepatic substance. On microscopic examination it could be seen that the small red regions were hemorrhages and that the number of patent sinusoids was decreasing gradually.

Within twenty to twenty-four hours the liver showed maximal toxic effects of carbon tetrachloride and appeared definitely enlarged, with a grayish surface. Microscopically the sinusoids seemed relatively few and far apart, since a large number of them had been obliterated. Those that remained active were somewhat tortuous with marked irregularity of the caliber of their lumens as a result of the encroachment of the swollen hepatic cells. The sinusoids supplied by the hepatic artery were more evident and more numerous than those supplied by the portal vein. The lobular pattern became somewhat distorted, and the draining veins, though prominent, were neither regular in their distribution nor central in their location as they were in the control rats which had normal livers. The hepatic cells were much enlarged and irregularly transparent and showed hazy borders. Some hepatic cells were so transparent that they looked like large vacuoles instead of cells containing cytoplasm. Globules of various sizes, resembling large leukocytes, could be seen scattered irregularly in the hepatic parenchymatous cells. Sections taken from such livers, frozen and stained for fat revealed the presence of fat globules of various sizes within the hepatic cells. Such sections also revealed that many irregularly scattered hepatic cells were extremely vacuolated.

Among rats that were observed several weeks after the single exposure to inhalation of carbon tetrachloride the livers had returned within two to four weeks to normal in color, structural pattern, intrahepatic vascular distribution and circulatory activity and could not be distinguished from livers of normal controls that had not received any carbon tetrachloride whatsoever. The single subcutaneous injection of 0.1 cc. of carbon tetrachloride gave the same intrahepatic changes as the half-hour exposure to carbon tetrachloride vapors.

*Effects of Repeated Exposure to Carbon Tetrachloride Vapors.*—Every few days, before and after the administration of carbon tetrachloride, a rat from each group was anesthetized and the intrahepatic circulation in the intact animal was studied. Whenever it was deemed advisable, specimens of liver were taken for sections which were stained for fat as well as with hematoxylin and eosin.

In the first six days the findings in the intrahepatic circulation were the same as those reported for the groups which had received only one dose of carbon tetrachloride.

About the end of the third week of daily exposure to carbon tetrachloride vapors, jaundice became apparent in most of the rats. Several of them stopped growing; a few lost weight rapidly, diarrhea developed and they died. The liver became pale gray, and its free borders were rounded. Numerous reddish areas could be seen throughout the gray hepatic surface. Microscopic examination with illumination of the liver in situ disclosed extreme disorganization of the lobular pattern with

decentralization of the central vein and collapse and obliteration of sinusoids. Regions of hemorrhage outside the sinusoids and around the central and interlobular vessels were noted. Regions of parenchymatous cells appeared moth-eaten, with debris of pigment and clumps of erythrocytes seen here and there in their midst. In some regions, the interlobular spaces became more prominent, but only the arterial branches showed definite blood flow. The lumens of the few patent sinusoids were irregular, and their blood supply was mainly arterial, supplied by interlobular branches which appeared sinuous in their course. Numerous leukocytes filled the meshes outside the blood vessels in the exaggerated interlobular spaces. Small nodules of various-sized hepatic cells grouped together in a haphazard manner projected here and there over the irregular surface of the liver. Very tortuous sinusoids receiving arterial blood ran through their meshes and finally drained into the neighboring veins which were located in the interlobular spaces. These were the central veins which had become eccentrically located as a result of injury and repair of hepatic parenchyma. The whole mass of disorganized hepatic cells and sinusoids closely resembled newly formed granulation tissues.

By the end of the third month of repeated administration of carbon tetrachloride, most of the surviving rats had become severely anemic, and in several of them ascites had developed in addition to their extreme loss of weight and jaundice. The surfaces of the liver had become extremely rough and granular, with irregularly rounded borders, and in several rats vascularized adhesions had developed between the inferior surface of the liver and the lesser curvature and anterior surface of the stomach and omentum. Microscopic study of the transilluminated liver showed extreme disorganization in the distribution of the intrahepatic vessels and their ramifications. The relatively few sinusoids were tortuous, and their lumens were irregular. One could see many more sinusoids supplied by arterial than by portal blood. Most of the vessels in these cirrhotic livers appeared much narrower than normal. No lobular pattern could be discerned. On microscopic examination of sections prepared from such livers, all the characteristics of typical cirrhosis were seen, namely, degenerated hepatic cells, disorganized arrangement of vessels with loss of the structural pattern of lobules, nodular growths of regenerated hepatic cells and increased growth of connective tissue, with leukocytic infiltration in the portal spaces and among the disorganized groups of hepatic cells.

## SUMMARY

Inhalation of carbon tetrachloride produces an immediate vasoconstrictor effect on the intrahepatic circulation of the rat but when it is continued for about half an hour, it produces pathologic changes in the

liver which become apparent in four to eight hours and maximal in twenty-four hours. During this period hepatic enlargement with fatty degeneration, hydropic changes and hemorrhages can be detected in the liver. At the same time the liver becomes anemic from obliteration of its vessels by the encroachment of the swollen hepatic cells on the vascular walls. Within two to four weeks after cessation of the administration of carbon tetrachloride the liver gradually returns to the state in which it was before administration.

Repeated inhalation of carbon tetrachloride produces cirrhosis, during the genesis of which important vascular changes can be observed. These vascular changes consist of an early and initial vasoconstriction during the inhalation of carbon tetrachloride and a prolonged secondary obliteration of the vascular channels, chiefly the venous ones, produced by the encroaching swelling and fatty degeneration of the hepatic parenchyma in the initial reactions. After the cirrhosis is advanced, the accumulation of debris and the growth of connective tissue lead to further collapse and obliteration of the vessels, especially the venous branches. Direct injury of walls of blood vessels with rupture of vascular radicles produces numerous small hemorrhages in the substance of the liver. The vascularization of the regenerated regions of hepatic parenchyma is mainly arterial and does not have any definite pattern. The lobular pattern is completely distorted, and the central veins are displaced. The large arterial and venous channels become very tortuous, especially the arterial ones, during the later stages of cirrhosis.

# DIETARY PRODUCTION OF HEPATIC CIRRHOSIS IN RABBITS

## WITH AN ANALYSIS OF THE FACTORS INVOLVED

M. A. SPELLBERG, M.D.

ROBERT W. KEETON, M.D.

AND

ROBERT GINSBERG, B.S.

CHICAGO

The cause of portal cirrhosis remains shrouded in obscurity. One point on which both clinical and experimental observations are in agreement is that alcohol is not the sole nor even the most important etiologic factor.[1] We recently reported the observation of fatty livers in guinea pigs that were subsisting on an apparently adequate diet, deficient only in ascorbic acid.[2] The addition of this factor did not prevent these changes in the liver.[2b] Extension of the experiments to rabbits showed that in this species the purely fatty changes pass into a stage of fibrous tissue proliferation and finally culminate in a cirrhosis resembling in many respects the so-called portal, Laennec's or "alcoholic" cirrhosis.

In this report data will be presented on the anatomic changes in the liver, chemical estimations of the total lipids in the liver, changes in the cholesterol, cholesterol esters and proteins of the serum and possible etiologic dietary factors.

### METHODS

The animals were of both sexes. Most of the guinea pigs weighed between 300 and 400 Gm. at the beginning of the experiments, while the rabbits weighed between 2.5 and 3 Kg. Some of the guinea pigs were larger, but this did not appear to influence the results.

The animals were given the thoroughly mixed, fresh basal diets and water ad libitum. The supplements of carrots and carrot greens were weighed out in the proportion of 2 Gm. per hundred grams of animal weight per day. Since these were readily and quickly eaten by the animals, they were simply put into the cages. The guinea pigs received pure ascorbic acid dissolved in water. This

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From the Department of Medicine, University of Illinois College of Medicine.

Presented before the Section on Pathology and Physiology at the Ninety-Second Annual Meeting of the American Medical Association, Cleveland, June 6, 1941.

1. (*a*) Meakins, J. C.: Practice of Medicine, St. Louis, C. V. Mosby Company, 1938, p. 720. (*b*) Bollman, J. L.: Ann. Int. Med. **12**:1, 1938. (*c*) Connor, C. L.: Am. J. Path. **14**:347, 1938. (*d*) Cates, H. B.: Arch. Int. Med. **67**:383, 1941.

2. Spellberg, M. A., and Keeton, R. W.: (*a*) Proc. Soc. Exper. Biol. & Med. **41**:570, 1939; (*b*) Am. J. M. Sc. **200**:688, 1940.

was given by mouth in the amount of 2 to 3 mg. per hundred grams of body weight per day. In the acute experiment the guinea pigs received the ascorbic acid for about 4 weeks and then were allowed to die from scurvy. Thus this experiment lasted usually from 6 to 8 weeks. In the chronic experiments the animals received the ascorbic acid throughout the experiment and consequently died from other causes. Since, as we [2b] have shown previously, the absence of ascorbic acid was not the cause of the fatty degeneration, the death of the animal from scurvy was simply used to abbreviate the experiment and yet have similarly treated controls. The synthetic vitamin K-like substances were given hypodermically. The alpha tocopherol was given by mouth; 100 mg. of pure alpha tocopherol was dissolved in 1 cc. of ethyl laurate, and 0.25 cc. or 25 mg. of vitamin was given twice weekly by mouth to each of the animals in this group.

Autopsies were made on the animals soon after death, and sections of the livers were stained by hematoxylin-eosin, sudan III, Mallory or Van Gieson stains. Samples were weighed out [2b] and used for chemical analysis of total lipoids by the Van Slyke [3a] manometric technic. The Van Slyke [3b] method was used also for the plasma protein analysis. The plasma cholesterol and esters were analyzed by the Schoenheimer-Sperry [4] modification of the Bloor technic. A photelometer was used in the color reading.

## OBSERVATIONS

*Anatomic Changes and Total Liver Lipid Content.*—In the guinea pig, as we [2] have shown previously, the most significant change is the fatty infiltration and degeneration of the liver. The amount of fat that is deposited in the liver bears an approximate relation to the length of time that the animal survives on the diet. Since true cirrhosis developed in only 1 guinea pig, it seems likely that in this species the stage of fibrous tissue proliferation is not so easily obtained.

In the rabbit the evidence is ample that the first stage is the same as in the guinea pig—fatty deposition in the liver. However, as the animals continue to survive, the liver lipids begin decreasing and fibrosis takes the ascendancy. The fat is apparently replaced by fibrous tissue, so that at death the amount of fat in the liver may be only slightly above normal. Thus one can see (table 1) that 4 animals that survived 103 to 147 days had a liver lipid range from 12.2 to 24.0 per cent, while those animals that died between the 123d and 228th day had a lipid range of 9.3 to 15.5 per cent, and the group with the longest survival time, 189 to 296 days, had a liver lipid content of 6.1 to 13.9 per cent. Significantly enough, the latter group had the greatest amount of fibrosis, presenting gross and microscopic pictures of portal cirrhosis. The group showing the greatest amount of fat had a minimal amount of fibrosis. This transition from fatty degeneration to cirrhosis is especially important, since it is thought that portal cirrhosis in the human being also goes through the fatty stage.

3. (*a*) Kirk, E. I.; Page, I. H., and Van Slyke, D. D.: J. Biol. Chem. **106**: 203, 1934. (*b*) Van Slyke, D. D.: ibid. **71**:235, 1927.

4. Schoenheimer, R., and Sperry, W. M.: J. Biol. Chem. **106**:745, 1934.

The gross and microscopic changes in the guinea pig livers are chiefly those of fatty metamorphosis. Since they were described elsewhere,[2b] no further discussion of them is indicated.

In the rabbits the anatomic changes in the liver depended on the length of time that the animals survived. If an animal died before 90 days from intercurrent infection, the liver was either slightly fatty or practically normal. If death occurred between 100 and 140 days, the liver was usually enlarged and yellow but quite smooth. Some resistance was evidenced on cutting, and microscopically increase of fat in the liver cells together with slight increase of fibrous tissue was noted. The animals that survived over 200 days almost invariably showed a liver with grossly irregular surfaces, and some, a liver that was definitely nodular. The nodules were coarsest and most prominent at the edge.

TABLE 1.—*Relation of Fibrosis to Fat Deposition in Livers of Rabbits on Experimental Diets*

| Rabbit | Diet | Days on Diet | Percentage of Fat in Liver | Fibrosis |
|---|---|---|---|---|
| RM5 | 1 | 281 | 9.8 | ++++ |
| RM7 | 1 | 206 | 6.1 | ++++ |
| 3RF2 | 3 | 189 | 6.2 | ++++ |
| RF10 | 1 | 296 | 13.9 | ++++ |
| RM13 | 1 | 167 | 15.5 | +++ |
| RM6 | 1 | 228 | 13.6 | +++ |
| 3RF4 | 3 | 209 | 11.4 | +++ |
| 18RF7 | 18 | 123 | 9.3 | +++ |
| 3RF6 | 3 | 121 | 17.0 | ++ |
| RF8 | 1 | 147 | 14.2 | ++ |
| RM9 | 1 | 103 | 12.2 | ++ |
| RF1 | 1 | 119 | 24.0 | + |

Even grossly one could detect septums of grayish white connective tissue surrounding the nodules. On cutting, the knife encountered resistance, and the cutting produced a grating sound. Some of these livers gave a cartilaginous sensation. Occasionally, one encountered an area measuring a square centimeter or more which was depressed and when cut appeared to be a fibrous strand.

Microscopically, the increase of fibrous tissue was striking. This tissue appeared to spread out from the portal areas, to envelop cords of liver tissue and thus to create pseudolobulation. In addition to the fibrosis and the fatty degeneration, actual necrosis was occasionally encountered. Islets of large eosinophilic parenchymal cells offered evidence that regeneration was going on side by side with degeneration. Round cell infiltration and dilatation of sinusoids were found along with the other abnormal findings. Some large bands of fibrous tissue were invaded with numerous multiplying bile ducts, the cells of which no doubt eventually gave rise to the regenerated liver tissue (figs. 1 and 2).

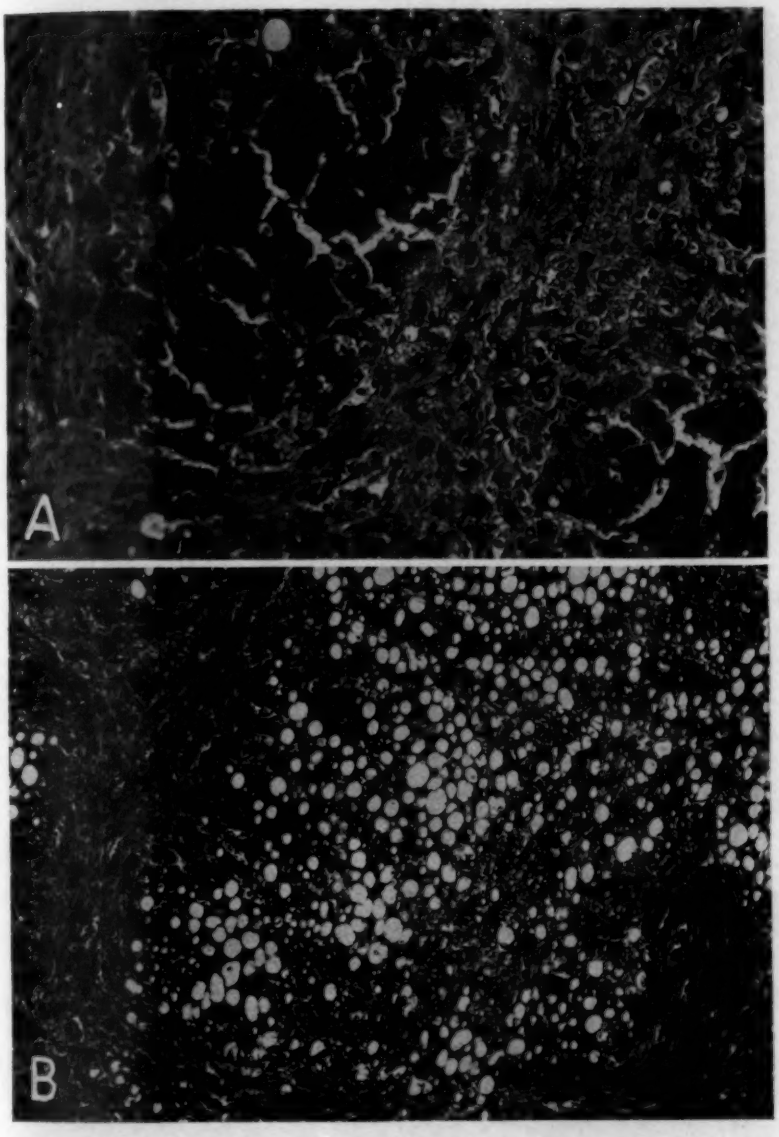


Fig. 1.—*A*, microscopic section of liver stained with eosin and hematoxylin, showing thick bands of fibrous tissue surrounding cords of liver cells with production of pseudolobulation. This section is from the liver of rabbit RM7 (table 1), fed diet 1 for 206 days; the value for the total liver lipids was 6.1 per cent. *B*, microscopic section of liver stained with eosin and hematoxylin, showing fatty degeneration of liver parenchyma, fibrosis and regenerating normal liver cells. The section is from the liver of rabbit RF10 (table 1), fed diet 1 for 296 days; the value for the total liver lipids was 13.9 per cent.

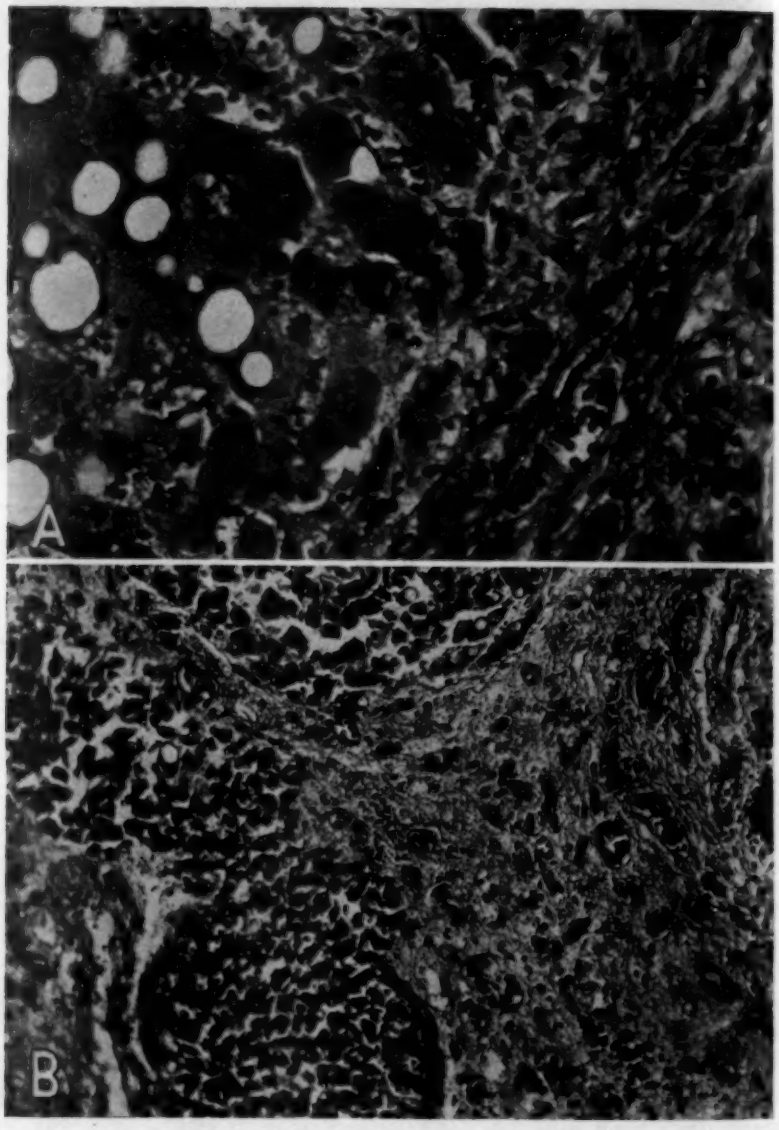


Fig. 2.—*A*, same as *B* in figure 1 but under higher magnification, showing the same changes in detail. *B*, microscopic section of liver stained with eosin and hematoxylin, showing a large strand of fibrous tissue invaded by newly formed bile ducts, and islands of liver cells. The section is from the liver of rabbit 3RF2 (table 1) on diet 3 for 189 days; the value for the total liver lipids was 6.2 per cent.

The most frequent other pathologic condition was pneumonia. This was the direct cause of death in most animals and was terminal in nature. It is interesting to note that this is the most frequent cause of death in human beings with cirrhosis (Evans and Gray [5]). Distention of the intestine and terminal diarrhea were also frequently encountered. The spleen was increased in size in some animals. Some atheromatous plaques were encountered in the aorta, but since we have no exact data on the occurrence of such lesions in nonexperimental animals, we are not in a position to stress the significance of this finding. Coccidiosis of the liver was encountered in 1 animal. Ascites of small degree was observed in 1 rabbit and 1 guinea pig. Changes in the fur of the rabbit were frequently noted.

TABLE 2.—*Values for Plasma Cholesterol and Cholesterol Esters in Normal Rabbits*

| Rabbit | Cholesterol, Mg. per 100 Cc. | | | Percentage of Esters |
|---|---|---|---|---|
| | Total | Free | Esters | |
| 1 | 230 | 65 | 165 | 71.7 |
| 2 | 200 | 67 | 133 | 66.5 |
| 3 | 166 | 63 | 103 | 61.3 |
| 4 | 236 | 103 | 133 | 57.0 |
| 5 | 140 | 54 | 86 | 61.5 |
| 6 | 178 | 85 | 93 | 52.3 |
| 7 | 190 | 70 | 120 | 63.2 |
| 8 | 120 | 37 | 83 | 69.1 |
| 9 | 152 | 53 | 99 | 65.1 |
| 10 | 174 | 50 | 124 | 71.2 |
| 11 | 186 | 64 | 122 | 65.6 |
| 12 | 118 | 37 | 81 | 68.9 |
| 13 | 132 | 43 | 89 | 67.4 |

*Chemical Changes in the Blood.*—(*a*) Plasma Cholesterol and Cholesterol Esters: The total cholesterol in the rabbit, as in the human being, varies a great deal, but the relative proportion of the esterified to the free cholesterol is remarkably constant. Thus the esters are between 52 and 71 per cent of the total plasma cholesterol in the normal rabbit (table 2). Only 2 of 13 normal animals examined had less than 60 per cent esters. In the experimental animals the highest was 44 and the lowest 7.7 per cent esters (table 3). Thus all the pathologic animals had less than 50 per cent of their plasma cholesterol in the ester form. Some of the animals listed in this table had their plasma cholesterol studied before and after they were put on the diet. Rabbit RM14 had 68.9 per cent esters before, and 23.8 per cent esters 88 days after, being fed the diet; rabbit 3RF5 had 65.1 per cent esters before, and 9.9 per cent esters 111 days after, being put on the diet. A drop in plasma

5. Evans, N., and Gray, P. A.: J. A. M. A. **110**:1159, 1938.

cholesterol esters has been accepted as a sign of damage to the liver.[6] Some investigators have also contended that the greater the "liver damage" the lower are the esters. The expression "liver damage" carried with it the connotation of anatomic changes in the liver. One is apt to imply, then, that only after the liver has been damaged does the free cholesterol–cholesterol ester ratio become reversed. The assumption that anatomic changes in the liver must occur before the cholesterol

TABLE 3.—*Values for Plasma Cholesterol and Cholesterol Esters in Experimental Rabbits*

| Rabbit | Diet | Cholesterol, Mg. per 100 Cc. | | Percentage of Ester | Days on Diet |
|---|---|---|---|---|---|
| | | Total | Ester | | |
| RM6 | 1 | 252 | 111 | 44.0 | 202 |
| RF8 | 1 | 126 | 46 | 37.0 | 83 |
| | | 250 | 105 | 42.0 | 124 |
| RM9 | 1 | 192 | 62 | 32.5 | 83 |
| RF10 | 1 | 386 | 36 | 9.4 | 96 |
| RM13 | 1 | 390 | 30 | 7.7 | 96 |
| | | 242 | 87 | 35.9 | 124 |
| RM14 | 1 | 118 | 81 | 68.9 | 0* |
| | | 109 | 26 | 23.8 | 88 |
| RM15 | 1 | 132 | 89 | 67.4 | 0* |
| | | 167 | 36 | 21.5 | 88 |
| 1SRF1 | 1S | 148 | 58 | 39.3 | 41 |
| | | 202 | 94 | 46.5 | 81 |
| 1SRF6 | 1S | 190 | 120 | 63.1 | 0* |
| | | 210 | 108 | 51.4 | 55 |
| 1SRF7 | 1S | 120 | 83 | 69.1 | 0* |
| | | 141 | 54 | 38.9 | 82 |
| 1SRF8 | 1S | 175 | 22 | 12.8 | 80 |
| 3RF2 | 3 | 244 | 39 | 15.9 | 52 |
| | | 262 | 51 | 19.5 | 85 |
| | | 139 | 26 | 19.0 | 208 |
| 3RF4 | 3 | 268 | 43 | 16.0 | 47 |
| | | 128 | 32 | 25.0 | 80 |
| | | 323 | 37 | 11.5 | 203 |
| 3RF5 | 3 | 152 | 99 | 65.1 | 0* |
| | | 311 | 30 | 9.9 | 111 |
| 3RF6 | 3 | 174 | 124 | 71.2 | 0* |
| | | 195 | 41 | 21.0 | 111 |

* The rabbit was on the normal diet prior to the beginning of the experiment.

changes take place is not borne out in this study. We note that in animal 3RF4, table 3, after 47 days on the diet the esters were 16 per cent of the total cholesterol and that in rabbit 3RF2 after 52 days the esters were 15.9 per cent of the total cholesterol. A significant drop is

6. (*a*) Epstein, E. Z.: Arch. Int. Med. **47**:82, 1931; (*b*) **50**:203, 1932; (*c*) Rev. Gastroenterol. **4**:12, 1937. (*d*) Epstein, E. Z., and Greenspon, H.: Arch. Int. Med. **58**:860, 1936. (*e*) Gardner, J. A., and Gainsborough, H.: Quart. J. Med. **23**:465, 1930. (*f*) Greene, C. H.; Hotz, R., and Leahy, E.: Arch. Int. Med. **65**:1130, 1940. (*g*) Myers, W. K., and Muntwyler, L.: Physiol. Rev. **20**:1, 1940.

also noted in animal 1SRF1 after 41 days. It is evident that these examples could be multiplied had we done more blood studies in the early phase of the experiment. Some of our rabbits that died even after up to 80 days on these experimental diets did not show any significant gross or microscopic changes in the liver except for a very slight increase of lipids. This suggests that the change in plasma cholesterol precedes the anatomic changes in the liver and may be indicative of the type of metabolic abnormality operative in this pathologic process.

(*b*) Plasma Proteins: The total plasma protein was normal in all animals except 1 rabbit, in which it was only slightly depressed to

TABLE 4.—*Values for Plasma Proteins and Albumin-Globulin Ratios in Experimental Rabbits*

| Rabbit | Diet | Total Protein, Gm. per 100 Cc. | Albumin, Gm. per 100 Cc. | Albumin-Globulin Ratio | Days on Diet |
|---|---|---|---|---|---|
| RM6 | 1 | 6.78 | .... | ... | ... |
| RF8 | 1 | 6.82 | 3.29 | 0.9 | 133 |
| RM14 | 1 | 8.19 | 4.72 | 1.4 | 0* |
| | | 6.04 | 4.48 | 2.8 | 91 |
| RM15 | 1 | 6.53 | 3.85 | 1.4 | 0* |
| | | 7.58 | | | |
| 1SRF6 | 1S | 6.47 | 3.78 | 1.4 | 38 |
| | | 5.75 | 3.78 | 1.9 | 55 |
| 1SRF7 | 1S | 5.85 | 3.81 | 2.0 | 85 |
| 1SRF8 | 1S | 7.75 | 4.38 | 1.3 | 85 |
| 3RF2 | 3 | 6.37 | 4.18 | 1.9 | 160 |
| 3RF4 | 3 | 6.04 | 4.12 | 2.1 | 75 |
| 3RF5 | 3 | 5.89 | 4.18 | 2.4 | 0* |
| | | 6.89 | 3.24 | 0.9 | 111 |
| 3RF6 | 3 | 5.07 | 3.90 | 2.2 | 0* |
| | | 4.63 | 3.01 | 1.8 | 111 |

* The rabbit was on the normal diet prior to the beginning of the experiment.

4.63 Gm. per hundred cubic centimeters of plasma (rabbit 3RF6, table 4). The albumin-globulin ratio was above 1.3 in all animals tested except 2. In these 2 animals (RF8 and 3RF5, table 4) this ratio was 0.9, a slightly subnormal value. It would be expected that the blood would mirror the predominant changes occurring in the liver. Thus, with the derangement of fat metabolism, it is not surprising that early changes in the cholesterol and cholesterol esters should occur in the blood stream. When the changes have progressed further, it might be assumed that the intermediary protein metabolism would be disturbed and that the proteogenic function of the liver would be damaged. At this point, alterations in the serum proteins should occur. It is possible that the blood was examined too early in the course of the experiment to permit detection of changes in the protein metabolism. Further studies

of animals in the terminal stages will be required before it can be concluded finally that this process does not depress the proteogenic function of the liver.

*Analysis of the Possible Etiologic Dietary Factors.*—(*a*) Vegetables: To investigate the possible relationship of various factors to this pathologic process, we used guinea pigs instead of rabbits. This was done because the fatty changes develop more quickly and valuable time is saved. The possibility that fresh vegetables, a normal constituent of the guinea pig diet, may contain some unknown factor necessary for liver function was tested by adding carrots and carrot tops to the basal diet (table 5). Diet 1 (table 7), the original diet, was used, and this was

TABLE 5.—*Percentage of Liver Lipids in Guinea Pigs on Various Diets (Chronic Experiment)*

| Diet 1 and Carrot Tops * | Diet 1 and Carrots * | Diet 3 (10% Yeast) | Diet 48 |
|---|---|---|---|
| 34.6 | 24.0 | 30.0 | 18.4 |
| 25.1 | 20.0 | 22.9 | 11.9 |
| 24.5 | 18.7 | 21.2 | 11.2 |
| 23.1 | 16.3 | 19.3 | 11.7 |

* The amount given was 2 Gm. of carrot tops or carrots per hundred grams of weight per day.

TABLE 6.—*Percentage of Liver Lipids in Rabbits on Synthetic Diet 5*

| Rabbit | Days | Percentage of Liver Lipids |
|---|---|---|
| 5RM1 | 56 | 3.45 |
| 5RF6 | 92 | 3.11 |
| 5RF7 | 44 | 3.08 |

supplemented by 2 Gm. of the vegetable per hundred grams of body weight per day. The animals seemed to thrive better with this supplement. Some of them survived over 300 days; others were put to death. All the animals on the carrot and carrot tops supplement showed severely fatty livers, 16 to 24 per cent fat in one group and 23 to 34 per cent fat in the other group (table 5).

(*b*) Yeast: The absence of the vitamin B complex from the diet has been mentioned as a possible cause of damage to the liver.[7] Rich and Hamilton [8] recently reported the production of hepatic cirrhosis in rabbits maintained on a diet supplemented with purified vitamins. When the entire B complex was given, the animals were protected, but when

7. György, P., and Goldblatt, H.: J. Exper. Med. **70**:185, 1939.
8. Rich, A. B., and Hamilton, J. D.: Bull. Johns Hopkins Hosp. **66**:185, 1940.

thiamine, riboflavin, nicotinic acid and pyridoxine were given instead, cirrhosis developed. We put a group of guinea pigs on a diet (3, table 7) modified by the addition of 10 per cent desiccated yeast before the last report appeared. These animals thrived well at first and survived 134 to 202 days, but all showed severely fatty livers, containing 19 to 39 per cent fat (table 5). Rabbits were put on this diet, and as will be noted by glancing at table 1, rabbit 3RF6 had a very

TABLE 7.—*Composition of Experimental Diets*

**Diet 1**
**(Approximately 20% fat, mostly butter)**

| | |
|---|---|
| Butter fat | 80 |
| Cod liver oil | 20 |
| Wheat bran and oats | 500 |
| Powdered whole milk | 300 |
| Salt (NaCl) | 10 |

**Diet 3**
(Approximately 20% fat, mostly butter)

Same as diet 1 except that 10% desiccated yeast is added

**Diet 4S**
(Approximately 5% fat)

No butter
Skimmed instead of whole milk

**Diet 1S**
(Approximately 13% fat, mostly butter)

Powdered skimmed milk instead of whole milk

**Diet 1 CR**
(Approximately 20% fat, mostly Crisco)

Butter, Milk fat, Cod liver oil — Replaced by — A hydrogenated vegetable oil (Crisco); Haliver oil concentrate (navitol), source of vitamins A and D

**Diet 4M**
(Approximately 12% fat, mostly butter)

Butter is replaced by wheat bran and oats

**Diet 1 CO**
(Approximately 20% fat, mostly cotton seed oil)

**Diet 5**

| | | |
|---|---|---|
| Dextrin | 300 | Same as diet 1 except that cotton seed oil replaces butter fat |
| Casein | 110 | |
| Crisco | 50 | |
| Salt | 40 | |
| Agar | 500 | (later reduced to 200) |

fatty liver, 17 per cent fat, and rabbits 3RF2 and 3RF4 had marked cirrhosis. The hepatic changes took place as easily in these animals as in the ones on diet 1, which contained no yeast supplement.

In order to test further the role of the B complex in this process, we maintained a group of rabbits on a diet which consisted of casein, hydrogenated cottonseed oil (Crisco) and dextrin in approximately the same proportions (table 7) as were used by Rich and Hamilton.[8] We added agar as a bulk-producing agent rather than rice as was done by the other workers. Carrots were not used. The Osborne and Mendel[9] salt mixture furnished a supply of minerals. Vitamins A and D were

9. Osborne, T. B., and Mendel, L. B.: J. Biol. Chem. **37**:557, 1919.

administered in the form of the concentrate navitol (Merck),[9a] 0.35 cc. twice a week to each animal. Vitamin E was given twice a week as alpha tocopherol, 12.5 mg. Thiamine hydrochloride per dose, 2 mg., nicotinic acid, 25 mg., and pyridoxine, 2 mg., were each given twice a week. Riboflavin, 5 mg., was given once a week.[10] All the vitamins were administered by mouth except thiamine hydrochloride, which was given subcutaneously. Three of these animals survived between 44 and 92 days, but none of them showed either a fatty or a fibrotic liver (table 6). In the report mentioned,[8] frank cirrhosis developed in the animals within as little as 25 days. In our experience the absence of the filtrate factors of the B complex does not result in cirrhosis. The absence of hepatic damage in these animals is probably due to the type and small amount of fat we used. This point will be discussed in greater detail later.

TABLE 8.—*Percentage of Liver Lipids in Guinea Pigs on Various Diets and Supplements (Acute Experiment)*

| Diet 1 | Diet 1 Plus Vitamin K | Diet 1 Plus Vitamin $K_5$ | Diet 1 Plus Vitamin E | Diet 1S | Diet 4M | Diet 4S | Diet 1CR | Diet 1C |
|---|---|---|---|---|---|---|---|---|
| 26.0 | 27.9 | 26.7 | 30.0 | 20.1 | 16.0 | 9.9 | 21.5 | 18.8 |
| 23.8 | 25.4 | 21.8 | 19.6 | 16.1 | 14.2 | 7.0 | 8.3 | 14.8 |
| 12.2 | 17.5 | 21.0 | 17.8 | 15.7 | 10.1 | 4.2 | 2.6 | 11.5 |
| 11.7 | 1.6 | 8.4 | 9.9 | 13.2 | .... | 2.2 | 2.0 | 7.1 |

(*c*) Vitamin K: While we were aware of the fact that in mammals vitamin K is formed by intestinal fermentation, we wanted to see the influence of an added supply of this factor on the changes in the liver. The diet itself (1) probably does not contain much of this vitamin. Using guinea pigs fed diet 1, we administered 2.5 mg. of synkayvite[11] (2-methyl-1, 4 dihydroxynaphthalene diphosphoric acid ester tetrasodium salt) subcutaneously twice a week to one group, and 4 amino-2-methylnaphthol,[12] 1 mg. three times a week, to another group. The animals were finally allowed to die from scurvy as did our controls fed diet 1. All animals except 1 showed a severe fatty condition of the liver (table 8), the fat content ranging as high as 28 per cent. One animal in the first group had a normal percentage of hepatic lipid. This animal did not eat the diet well and was the first one to die. We think this single exception is not significant.

9a. Navitol is stated to contain in 1 cc. 55,000 U. S. P. units of vitamin A and 10,000 U. S. P. units of vitamin D.

10. The alpha tocopherol, riboflavin and pyridoxine were donated by Merck & Company, Inc.

11. The synkayvite was donated by Hoffmann-La Roche, Inc.

12. The 4-amino-2-methylnaphthol was donated by Parke Davis & Company.

(*d*) Vitamin E: Because vitamin E was not very abundant in our diet and for the sake of completeness, we administered 25 mg. of alpha tocopherol in 0.25 cc. ethyllaurate twice a week by mouth to a group of guinea pigs. It can be seen by referring to table 7 that fatty liver developed in all these animals.

(*e*) Variation in Amount and Type of Fat: Our original diet 1 (table 7) contains about 20 per cent fat, mostly butter fat. Although this cannot be considered a high fat diet, we nevertheless reduced the fat by substituting skimmed, powdered milk for whole milk (diet 1S, table 7). This reduced the amount of fat to approximately 13 per cent. In diet 4M the butter was replaced by wheat bran and oats. This appreciable reduction in the dietary fat and coincidental increase in the other constituents did not influence significantly the liver lipids of our experimental animals; on diet 1S the liver lipids were from 13 to 20 per cent, and on diet 4M, 10 to 16 per cent. The latter is a result on 3 animals and may represent a slight reduction. In diet 4S (table 7) we further reduced the dietary fat to about 5 per cent by excluding butter and using skimmed instead of whole milk. The liver fat in these animals was 2.2 to 9.9 per cent, which shows marked reduction as compared with the results on the other diets, and is nearly normal. In guinea pigs on normal diets the liver lipids were usually 2 to 4 per cent; an occasional animal showed 7 per cent fat; so in this experiment only 1 animal had a hepatic lipid content slightly above what may be found normally.

The experiments just described were acute; i. e., the animals survived 6 to 8 weeks and were allowed to die from scurvy. We also used diet 4S in a chronic experiment, in which the animals survived 138 to 163 days (table 5). These animals showed livers moderately fatty, 11 to 18 per cent fat, but much below the livers of the other animals in the chronic experiments. On diet 3, for example, the liver was 19 to 39 per cent fat.

To ascertain the role of the type of fat, we modified our diet by replacing the butter fat with cottonseed oil (diet 1CO, tables 7 and 8). On this diet the livers were from 7.1 to 18.8 per cent lipids, showing perhaps a slight reduction but still quite fatty. In another diet we used the hydrogenated vegetable oil Crisco. The principal oil used in the manufacture of this is cottonseed oil. The iodine number of cottonseed oil is about 110, but the iodine number of this fat is about 73. In diet 1CR we replaced the 20 Gm. of cod liver oil by 2 Gm. of navitol and 18 Gm. of the hydrogenated vegetable oil. Three animals on this diet had hepatic lipid values of 2.0, 2.6 and 8.3 per cent, which are nearly normal values, and only 1 animal had a fatty liver, 21.5 per cent fat. It should be stressed that this diet contained the same amount of fat

(20 per cent) as diet 1, and yet on the latter diet 2 animals had over 23 per cent fat and 2 had 11 and 12 per cent fat in the liver. Of 16 animals on the butter-containing diet, only 1 had a normal percentage for liver lipid and 2 had 8.4 and 9.9 per cent, while the rest had 11.7 to 30 per cent fat.

Our diet containing 20 per cent fat is not a very high fat diet compared with the high fat diets that have been used by other experimenters. The diets used in producing fatty livers in rats contained 40 per cent lard, in addition to being poor in proteins.[13] Chaikoff and Connor [14] used a diet containing about 90 per cent fat. This diet produced cirrhosis of the liver in dogs when fed to them for a sufficiently long period. Bollman [15] used diets containing as much as 80 per cent fat. Since the Crisco-containing diet, although having the same or a larger proportion of fat, was not as efficacious in producing a fatty condition of the liver in the guinea pigs as were the butter-containing diets, it seems that the type of fat was even more a factor than the amount of fat. That the liver serves an important role in fat transport and metabolism in the animal body is a well known fact, although its exact functions are unknown.[16] It is thought that the manner in which choline acts to cause mobilization of fat from the liver is that of aiding phosphorylation, one of the constituents of phospholipids being choline. The latter compound is a vehicle by which fat is transported from the liver, the other being, probably, esterified cholesterol.[16] It should also be pointed out that neither choline nor lipocaic influenced the fatty changes in our animals.[2b] The drop in cholesterol esters in our animals was an indication of impairment of this function. The liver also desaturates fatty acids and possibly makes other chemical alterations in the fats consumed, to adapt them to the given species. Our results make us wonder whether the introduction into the liver of sizable amounts of certain fats which are metabolized with difficulty is not the important factor. These, once anchored there, would by their presence set up irritation, which would eventually result in cirrhosis. It is not improbable that a similar mechanism may be operative in the causation of human cirrhosis.

We also attempted to use lard in one of our diets, but 8 animals died in 10 to 14 days with a precipitant loss of weight, which suggested that the animals refused to eat the diet and starved to death. We therefore

13. (*a*) Aylward, F., and Holt, L. E.: J. Biol. Chem. **121**:60, 1937. (*b*) Best, C. H., and Ridehout, J. H.: Am. J. Physiol. **122**:67, 1938.

14. Chaikoff, I. L., and Connor, C. L.: Proc. Soc. Exper. Biol. & Med. **43**:638, 1940.

15. Flock, E. V., and Bollman, J. L.: Proc. Soc. Exper. Biol. & Med. **36**:853, 1937. Bollman.[1b]

16. Bloor, W. R.: Physiol. Rev. **19**:557, 1939.

have insufficient data on this diet. Rats that subsisted on the butter-containing diet for months did not suffer any noteworthy changes in the liver. This observation suggests a species difference.


## SUMMARY

Cirrhosis of the liver was produced in rabbits and fatty liver in guinea pigs when the animals were fed an apparently adequate diet containing approximately 20 per cent fat, chiefly butter fat.

Supplements of desiccated brewers' yeast, alpha tocopherol, synthetic vitamin K–like substances and 2 Gm. of vegetables per hundred grams of body weight did not lessen the fatty degeneration.

A reduction of the fat in the basal diet to 5 per cent by exclusion of butter and use of skimmed milk caused a marked reduction in liver lipids.

A replacement of butter fat with cottonseed oil did not cause a decrease in the liver fat, but the substitution of a hydrogenated vegetable oil (Crisco) caused a significant drop in liver fat.

It is suggested that the liver may have difficulty in metabolizing certain types of fats. When these become anchored in the hepatic parenchyma, permanent damage results. There was an early drop in plasma cholesterol esters in the experimental rabbits.

There was no significant change in serum proteins observed in rabbits which had been maintained on the diet 100 days. Data are not available on animals which had been fed the diet a longer period.


## ABSTRACT OF DISCUSSION

THOMAS E. MACHELLA, Philadelphia: Feeding a basal diet containing 20 per cent butter fat, Dr. Spellberg and his associates observed the development of fatty changes in the livers of guinea pigs and rabbits and of cirrhotic changes in the livers of 1 guinea pig and several rabbits. In 1940 Rich and Hamilton reported the development of cirrhosis in rabbits maintained on a diet containing 20 per cent fat in the form of the hydrogenated vegetable oil Crisco or of lard. They held that a deficiency of some factor present in the vitamin B complex, and not the fat, was responsible. These contentions are the direct opposite of those which Dr. Spellberg has just presented. His inability, as well as that of Maguire and myself when we maintained rats on a diet deficient in vitamin B complex, to confirm the observations of Rich and Hamilton raises the question whether or not some factor other than the diet might be responsible for the development of the hepatic changes observed in rabbits by both groups. Coccidia infestation, not uncommon in rabbits, is able to incite most of the microscopic changes described, and for this reason many laboratories refuse to use rabbits in studies involving the experimental production of cirrhosis. Failure to recognize this pitfall was responsible for the confusion in the literature which arose when the role of alcohol in the production of human cirrhosis was first being investigated. In this connection it is interesting that Rich and Hamilton, and also Dr. Spellberg and his associates, were able to isolate coccidia from their animals. I should like to ask Dr. Spellberg whether

the 1 animal from which coccidia were isolated was a rabbit or a guinea pig; guinea pigs are infested less frequently.

The role of fat in the development of cirrhosis certainly needs investigation. We know that a liver rich in fat and poor in protein and glycogen is more susceptible to damage by hepatotoxic agents and also that the reverse is true. One of the first signs of damage to a hepatic cell which can be detected by the usual staining technics is the accumulation of fat droplets in the cell, but it is not always possible to differentiate this microscopic appearance from that which develops normally after the ingestion of fat in excess.

The failure of Dr. Spellberg's rats to show evidence of hepatic damage when fed the same diet that produced hepatic damage in rabbits and guinea pigs is especially interesting in view of the recently published preliminary report of observations on rats by György and Goldblatt. These workers observed necrosis with or without cirrhosis in animals fed a diet containing 20 per cent fat in the form of lard more regularly than in those fed a diet containing 8 per cent fat in the form of butter. It will be recalled that Dr. Spellberg's diet contained a much higher percentage of fat in the form of butter. At present a critical evaluation of the work of György and Goldblatt awaits detailed publication of their experiments.

Finally, it would seem that, as Dr. Spellberg and his associates continue their work, it would be more pertinent to the problem at hand if they should confine their attempts to analyze the possible factors responsible for the development of cirrhosis in rabbits to that species and not include guinea pigs, especially since cirrhosis developed in only 1 of the guinea pigs. Also, data dealing with the daily food consumption of each animal might reveal why the livers of some animals contain more or less fat on the basal diet if the type of fat is altered. The amount of the diet consumed by each animal might well explain the variations.

M. Laurence Montgomery, San Francisco: Dr. Spellberg asked a representative of the laboratory of the University of California Hospital to discuss his work. We are at some disadvantage because we have not had much experience with this type of production of fatty livers. Our work has been exclusively with dogs using depancreatized and duct-ligated preparations. Yet there are a few matters of interest I feel I can contribute to the discussion.

One is that Dr. Keeton and Dr. Spellberg, together with many others, are in the process of gathering a tremendous volume of material which will be of great use to pathologists in the future, when they are able to sift and analyze it. Until then many of the questions that are in his mind must remain unanswered.

There are so many causes of fatty infiltration of the liver that one is inclined to believe the process is simply a natural response of the liver cell to nonspecific injury. The fact that the damage is done by so many different substances indicates that one must be cautious in attributing specificity of response to any one of them.

The blood cholesterol changes that he observed are interesting, both in their timing and in their relation to fatty liver, because they are similar to those that occur in the depancreatized or duct-ligated dog in which fatty liver develops. We found that within three weeks after pancreatectomy the blood fats begin to fall, whereas it takes many weeks for fatty liver to develop. We have wondered in the past whether the two phenomena were associated with the nature of the dietary fat.

The observation of Keeton and Spellberg that butter fat somehow differs from other fat in relation to this process is of interest because of the suggestion that

the differences between the observations from our laboratory and those from others on the depancratized dog may be due to the fact that this constituent is present in some diets and not in others. I believe we should keep this clearly in mind.

Dr. Spellberg and Dr. Keeton have a lot of interesting work to do, and as they carry it on, we should learn a great deal more about these particular animals and their response to the high fat diets to which they usually are not accustomed.

V. H. Moon, Philadelphia: I have been deeply interested in the paper presented by Dr. Spellberg. Years ago my associates and I made a complete survey of all experimental cirrhosis found described in the literature. We came to the conclusion that cirrhosis can best be interpreted as chronic diffuse hepatitis and that its causes will be found in those agents or combinations of agents which will produce chronic injury, followed by inflammation of the liver.

The agents which have been used successfully in the production of one form or another of cirrhosis were capable of causing necrosis of hepatic cells. Among these were phosphorus, tars, arsenic and others, each of which when fed to animals in appropriate doses produced necrosis of liver cells. If the injury was continued, this process was followed by inflammation and cirrhotic changes.

I was interested particularly in the suggestion that irritating products of fat metabolism or catabolism caused the changes described in these experiments. This would supply another instance of an irritating substance producing degeneration of liver tissue followed by fibrosis.

I should like to ask Dr. Spellberg whether in some of his early experiments—those which were terminated before cirrhosis developed—there was evidence of necrosis and of inflammatory changes in the liver as a precirrhotic process.

It is understood, of course, that various conditions will reduce the resistance of the liver to damaging agents. One of those conditions is represented by a low glycogen content of the liver, as seen in patients with chronic exophthalmic goiter or hyperthyroidism. Another that reduces resistance is the loading of the liver with fat. It is interesting in these experiments that the loading of the liver with fat, perhaps accompanied by irritating products of fatty metabolism, may have produced the conditions essential for the development fo cirrhosis, that of prolonged damage followed by fibrotic changes.

J. P. Simonds, Chicago: I should like to ask Dr. Spellberg whether these animals were fed with known butter fat or with butter bought in the market and probably containing dyes to render the color uniform. Certain dyes used in coloring butter are known to produce pathologic changes in the livers of animals. Has this possibility been excluded?

M. A. Spellberg, Chicago: In regard to the question of Dr. Machella, one of our rabbit livers was infested with coccidia. The pathologic appearance of this liver was entirely different from that in the cirrhotic livers; i. e., the coccidia infestation is localized to the biliary passages, and there are a tremendous hyperplasia of the epithelial lining of the bile ducts with villus formation and dilatation of the lumen. The fibrosis is around those tremendously dilated hyperplastic bile ducts and does not involve so much the liver parenchyma. That particular pathologic condition was in an early stage; i. e., the animal was not on the diet for a sufficient length of time. If coccidia were an important factor here, we should expect the animals, even those with the infestation in the early stages, or some of our normal animals, to show the same pathologic picture, whereas such was not the case. Moreover, early there were always the fatty changes and practically no fibrosis

or very little. The fibrosis took place later in the experiment. The guinea pigs, in which fatty changes develop also, are admittedly not so subject to coccidia infestation.

A point I think should be stressed is the one that we used the same butter-containing diet in experiments on rats, and the diet produced no damage in the livers of these rats.

The recent experiments by György and Goldblatt in which they tried to produce cirrhosis of the liver in rats by means of a diet deficient in vitamin B complex were not successful while they used a butter-containing diet. However, when they changed the butter fat to lard, they were more successful. That brings out the point that I tried to stress, that there may be a species difference. In the guinea pig and in the rabbit butter fat may be the toxic lipid, whereas in the rat lard may be toxic. We also used lard in some experiments on guinea pigs. However, the guinea pigs did not eat the diet. The food was apparently not very palatable, and a great may starved to death. A few survived, but not enough of them to make our results conclusive. But it is suggestive that on the contrary lard may not produce fatty liver in guinea pigs as readily as butter.

The interesting correlation there is that lard and the hydrogenated vegetable oil Crisco have practically the same degree of saturation and that there may be some correlation between the degree of saturation of the fat and the particular species in which the fat may be toxic.

The other point brought up was that we should use rabbits instead of guinea pigs in these experiments. We agree as to that. We are transferring our experiments to rabbits, but guinea pigs showed the definite fatty changes much earlier than rabbits, and in order to save time we used guinea pigs as a sort of preliminary experiment to point the way to what we might use in rabbits more successfully.

As far as consumption of the diet is concerned, the means that we used to gage whether the animal consumed the diet properly or not was the gain in weight. Most of our experimental animals, including those on the Crisco diet, which did not show fatty liver, gained weight similarly to the ones on the butter-containing diets.

Dr. Moon's question can be answered in the affirmative. We did notice both necrosis and inflammatory changes. The latter were demonstrated by leukocytic infiltration in various regions of the liver.

In regard to the last question, we used butter, not butter fat. However, if the butter contains sufficient toxic substances to produce these changes in animals, it looks as if the Pure Food and Drug Act ought to be brought to bear on the manufacturers of butter. There was 16 per cent butter fat in the original diet, but only 8 per cent of this was derived from butter; the rest was from the milk fat, which of course contains no pigment. So the pigment, if any, was infinitesimal.

Moreover, fatty liver was also produced by the cottonseed oil–containing diet. So I doubt very much whether the changes were due to the pigment in the butter. Our experiments may eventually lead to the conclusion that there are certain food substances which have been considered innocuous for the human being but which if consumed in sufficient amounts may result in damage to the liver, degeneration and cirrhosis.

# DRAINAGE OF THE PULMONARY VEINS INTO THE RIGHT SIDE OF THE HEART

HENRY BRODY, M.D.
NEW YORK

Anomalous drainage of all or part of the lungs into the vessels of the major venous system is relatively uncommon. No complete collection of the recorded cases is available. The first report of a case, in which there is described a single vein from the upper lobe of the right lung emptying into the superior vena cava, is ascribed to Winslow in 1739. I have been able to collect from the literature 100 cases [1] and have seen 2 others, which are here reported. Of these, including the 2 here reported, 37 are instances of total drainage of the lungs into the right atrium or its tributaries. The drainage of a single pulmonary vein, or those from only one side, into the right side of the heart is not incompatible with normal life, and many examples of it have been discovered at the anatomy dissecting table in the medical school.

The cases of incomplete drainage into the right side of the heart or its tributaries are listed in table 1. Since Winslow's case, I have collected 64 others. The embryologic defect involves the right vessels about twice as frequently as the left. In 2 instances, right and left pulmonary veins drained into the lesser circulation. One of the subjects was a child (age not given) and the other a young adult (22 years). Incomplete drainage is not incompatible with long life. In the case reported by Dean and Fox this finding was observed in an 86 year old person. In 35 of the collected cases the patients lived to adulthood. In 19 instances the age was not given, but in regard to all but a few of these the presumption is that the patients were adults. Nine patients died before the age of 1 year, and 2 of these showed associated cardiac anomalies. The commonest site into which drainage occurs is the superior vena cava (29 cases); the second commonest is the right atrium (18 cases), and this is associated with the more common involvement of the right pulmonary vessels; the left innominate vein is the third most frequent site (14 cases). The coronary sinus, inferior vena cava, azygos vein and left subclavian vein are the less frequent sites.

From the Department of Pathology, Beth Israel Hospital.

1. The only similar anomalies in species other than man of which I have found records without carefully searching the zoologic literature are the several reported by Warren in the frog. He found pulmonary veins communicating with the hepatic portal and renal portal systems.

TABLE 1.—*Incomplete Drainage of the Pulmonary Veins into the Right Atrium or Its Tributaries*

| Author * | Sex | Age | Foramen Ovale | Ductus Arteriosus | Drainage | Comment |
|---|---|---|---|---|---|---|
| Winslow † | .. | ..... | ..... | ..... | Right pulmonary vein into superior vena cava | |
| Weese | .. | ..... | ..... | ..... | Right upper into right atrium | Complicated cardiac anomalies |
| Breschet † (1) | .. | ..... | ..... | ..... | Right into superior vena cava | |
| (2) | .. | ..... | ..... | ..... | Right into right atrium | |
| Meckel † | .. | ..... | ..... | ..... | Right upper into superior vena cava | |
| Weber | M | 60 years | ..... | ..... | Left into left subclavian vein | |
| Cooper | .. | 10 months | Open | Closed | Right into inferior vena cava | |
| Chassinat | F | 19 days | Open | Probe patency | Right into inferior vena cava | Dextroposition of aorta; interventricular septal defect; atresia of pulmonary artery |
| Hyrtl † | .. | ..... | ..... | ..... | 5 or 6 small veins from left upper lobe into rudimentary left vena cava | |
| Stoltz † | .. | 3 days | ..... | ..... | Right into right atrium | |
| Lacroix | M | 43 years | Open | ..... | Right into right atrium | Large interatrial septal defect |
| Guillabert † | .. | ..... | ..... | ..... | Right upper into superior vena cava; right middle of azygos | |
| Gegenbaur | .. | ..... | ..... | ..... | Right upper into superior vena cava | |
| Lambl | F | 12 years | Open | ..... | Right into right atrium | |
| Duchek † | M | Adult | ..... | ..... | Right upper into left superior vena cava | |
| d'Albini | .. | Adult | ..... | ..... | Right upper into superior vena cava | |
| Hickman | F | 28 years | Closed | ..... | Right into coronary sinus | Situs inversus |
| Gruber (1) | M | Adult | ..... | ..... | Right upper into superior vena cava | |
| Chiari | M | 2 years | Open | Closed | Right into right atrium | Right side of heart larger than left |
| Lorens | M | 3 days | ..... | Open | Right into right atrium | Almost complete absence of interatrial septum |
| Gruber (2) | M | Adult | ..... | Closed | Right upper into superior vena cava | |
| Rokitansky | .. | ..... | ..... | ..... | Right into right atrium | |
| Hepburn | M | Adult | Closed | Closed | Right into superior vena cava | Defect in interatrial septum |
| Shepherd | F | 30 years | ..... | ..... | Right into azygos vein | Arterial anomalies |
| Bachhammer † | .. | ..... | ..... | ..... | Left into left innominate vein | |
| Bardeleben † | .... | ..... | ..... | ..... | Right upper into superior vena cava | |
| Etlinger | F | 4 months | Open | Open | Right into right atrium | Interventricular septal defect; dextroposition of aorta; atresia of pulmonary artery |
| Theremin † | .. | 2 months | ..... | ..... | Left into left superior vena cava | |
| Blair | M | Adult | ..... | ..... | Left upper into left innominate vein | |
| Thane | .. | ..... | ..... | ..... | Left upper into left innominate vein | |
| Ingalls | .. | Adult | Closed | ..... | 2 of 3 right into superior vena cava | Superior vena cava emptied partly into right, partly into left atrium |

| | | | | | | |
|---|---|---|---|---|---|---|
| Stoeber | .. | Child | Open | Closed | Right upper into right atrium; left upper into right atrium | Horizontal septum divided left atrium; upper chamber received right and left lower pulmonary veins, and lower received the foramen ovale |
| Looten and Ruyssen | .. | ..... | ..... | ..... | Left upper into left innominate vein | |
| Park | M | 2 months | ..... | ..... | Right into inferior vena cava | |
| Geddes | .. | Adult | Open | ..... | 4 of 5 right into superior vena cava | Superior vena cava emptied partly into right, partly into left, atrium |
| Gérard and Leroy | F | 71 years | ..... | ..... | Right upper and middle into superior vena cava; right lower into right atrium | Interventricular septal defect (5 × 3 mm.) |
| Merkel (Nützel) | M | 47 years | ..... | ..... | Right upper into superior vena cava | Case also reported by Nützel |
| Patterson | .. | Adult | ..... | ..... | Left into left innominate vein | |
| Gérard | M | 70 years | Closed | ..... | Right upper into superior vena cava | Interventricular septal defect; case also reported by Marissal |
| Johnston | M | 56 years | Open | ..... | Left upper into left innominate vein | Perforation in fossa ovalis |
| MacCready (1) | .. | Adult | ..... | ..... | 1 of 2 left into left innominate vein | |
| (2) | .. | Adult | ..... | ..... | 1 of 2 left into left innominate vein | |
| (3) | .. | Adult | ..... | ..... | Right upper into superior vena cava | |
| Olivier | .. | Adult | ..... | ..... | Left into innominate vein | |
| Cords | M | 34 years | Slit | Closed | Left upper into left innominate vein | |
| Ratner, Abbott and Beattie | .. | ..... | ..... | ..... | Left into left side of single atrium | Situs inversus; cor triloculare biventriculare; other anomalies |
| George | .. | ..... | ..... | ..... | Left upper into left innominate vein | |
| Dean and Fox | .. | 86 years | ..... | ..... | Left into left innominate vein | |
| Jack and Lee | .. | Adult | ..... | ..... | Left upper into left innominate vein | |
| Curth | M | 64 years | ..... | ..... | Right upper into superior vena cava | |
| Paul | M | 25 years | Slit | Closed | Right upper into superior vena cava; right middle and lower into right atrium | |
| Van Cleave | M | 50 years | ..... | ..... | Right upper and middle into right atrium | Interatrial septal defect |
| Barge | F | 4 months | ..... | ..... | Right upper into superior vena cava | Complicated cardiac anomalies |
| Kolesnikow | .. | ..... | ..... | ..... | Right upper into superior vena cava | |
| Lukjanov † | .. | ..... | ..... | ..... | Right upper into superior vena cava | |
| Adachi (1) | M | 57 years | ..... | ..... | Right upper into superior vena cava | |
| (2) | M | 22 years | Open | ..... | 2 of 5 right pulmonary veins into superior vena cava; 2 of 5 into right atrium | |
| Freerksen | M | Adult | ..... | ..... | Left upper into left innominate vein | |
| Mildenberger | M | 4 months | Open | Open | Right into right atrium | |
| Fink | .. | Adult | Closed | Closed | Left into left innominate vein | |
| Roberts (1) | F | 17 years | ..... | ..... | Right into right atrium | Tetralogy of Fallot |
| (2) | M | 44 years | ..... | Open | Left into coronary sinus | Interventricular septal defect; coarctation of aorta |
| (3) | F | 3 years | ..... | ..... | Into coronary sinus | |
| Lightner | .. | ..... | ..... | ..... | Right into right atrium | Essentially cor biloculare |
| Atkinson and coworkers | M | 67 years | Closed | ..... | 2 upper of 4 right pulmonary veins into superior vena cava | Interatrial septal defect; superior vena cava communicates with both atria |

* The table is arranged in chronologic order.
† The original source was not consulted.

TABLE 2A.—*Total Drainage of Pulmonary Veins into the Right Atrium or Its Tributaries, Without Other Major Cardiac Anomalies*

| Author * | Sex | Age | Foramen Ovale | Ductus Arteriosus | Drainage Into | Comment |
|---|---|---|---|---|---|---|
| Friedlowsky | .. | 18 days | Open | Open | Left innominate vein | |
| Chaffey | M | 11 weeks | Open | Probe patency | Left innominate vein | Left atrium rudimentary; left ventricle small |
| Geipel † | F | Fetus | ..... | ..... | Persistent right duct of Cuvier | 5th-6th fetal month |
| Nabarro | F | 5 months | Slit | Closed | Coronary sinus | Left atrium and ventricle small |
| Kera † | F | 27 years | Open | Partly closed | Right atrium | Left atrium small |
| Ghon | M | 15 days | Slit | Probe patency | Portal vein | |
| Ono | F | 36 days | ..... | Open | Superior vena cava | |
| Schleussing | M | 2 months | Open | Open | Left innominate vein | |
| Uchida † | .. | Fetus | ..... | ..... | Inferior vena cava | Partial situs inversus; 8th fetal month |
| Rebensberg | M | 3 months | Open | Closed | Right atrium and superior vena cava. | Right side of heart larger than left |
| Munck | M | 3 months | Open | Open | Portal vein | Ductus arantii closed; left atrium small; left ventricle smaller than right |
| Grosse | M | 3.5 years | Open | Closed | Left innominate vein | |
| DeGroat and Thatcher | F | 2.3 years | Open | "Absent" | Coronary sinus | Left ventricle exceptionally small |
| Engels | F | 6 months | Open | Closed | Right atrium | |
| Nagel | F | 84 days | Slit | Closed | Coronary sinus | |
| Gander | M | 2 months | Open | ..... | Left innominate vein | Left side of heart small |
| Duff | M | 9 months | Open | Closed | Right atrium | Left atrium diminutive; left ventricle small |
| Terplan and Sanes | M | 5 months | Slit | Closed | Coronary sinus | Left atrium and ventricle small |
| Palmer (1) | M | 6 months | Open | Closed | Coronary sinus | Left atrium and ventricle very small |
| (2) | F | 3 months | Open | Open | Coronary sinus | Left atrium and ventricle small |
| (3) | M | 16 months | Open | ..... | Left innominate vein | |
| Brody (1) | F | 10 weeks | Slit | Closed | Coronary sinus | Left atrium and ventricle small |
| (2) | M | 10 weeks | Slit | Probe patency | Superior vena cava | Left atrium and ventricle small |

* The table is arranged in chronologic order.

† The original source was not consulted.

TABLE 2B.—*Total Drainage of Pulmonary Veins into the Right Atrium or Its Tributaries, Associated with Other Major Cardiac Anomalies*

| Author | Sex | Age | Foramen Ovale | Ductus Arteriosus | Drainage Into | Associated Anomalies |
|---|---|---|---|---|---|---|
| Wilson | .. | 7 days | ..... | ..... | Superior vena cava | Cor biloculare |
| Ramsbotham | .. | Newborn | ..... | Open | Left into subclavian vein; right into portal vein | Cor biloculare |
| Bochdalek | M | 4 days | ..... | Closed | Right upper into superior vena cava; remainder of pulmonary veins into portal vein | Inferior vena cava opens half into each atrium |
| Arnold | F | 15 weeks | ..... | Open | Portal vein | Right atrium received two hepatic veins; left atrium received both venae cavae and coronary sinus; cor triloculare biatrium |
| Rokitansky | M | 19 years | ..... | Closed | "Right" atrium | Essentially single-atrial heart |
| Epstein | F | 1 month | Open | ..... | Superior vena cava | Partial defect of interatrial septum; defect of interventricular septum; transposition of aorta; hepatic veins empty into left atrium |
| Miura | F | 6 months | Open | Open | Azygos vein | Superior vena cava emptying into left atrium and azygos vein into right atrium; left atrium and ventricle small |
| Audry and Lacroix | .. | 2 days | ..... | ..... | Right atrium | Large interventricular septal defect; transposition of aorta |
| Probyn-Williams | .. | 1 month | ..... | Open | Incomplete autopsy; course of pulmonary veins unknown | Single atrium with incomplete septum, receiving only a single vessel, the superior vena cava |
| Schelenz | M | 3 years | Open | ..... | Right innominate vein | Situs inversus; interventricular septal defect; transposition of aorta; inferior vena cava absent, left azygos vein substituting for it; hepatic veins enter atriums |
| Schröder | M | 26 days | ..... | ..... | Right innominate vein | Absence of interatrial septum and coronary sinus; pulmonary stenosis |
| Jensen * | .. | ..... | ..... | ..... | Right into superior vena cava; left into innominate vein | Cor biloculare |
| Wood and Williams | F | 15 years | ..... | Open | Right side of single atrium | Cor biloculare; pulmonic atresia |
| Davies and MacConaill | M | 4 days | ..... | Enlarged | Persistent sinus venosus | Cor biloculare; persistent sinus venosus; atresia of aorta |

* The original source was not consulted.

Complete drainage of blood from the pulmonary bed into the right side of the heart is encountered much less frequently. It would not be expected a priori that such drainage would be compatible with life beyond early infancy. In fact, it might be wondered how this condition could permit postnatal life at all. Of the 37 patients (including the 2 whose cases are here reported), however, 8 lived beyond 6 months of age. Their ages were 9 months, 16 months, 2⅓, 3, 3½, 15, 19 and 27 years. Of the last 3, the first had cor biloculare, which strictly does not belong with this series; the second (Rokitansky's case), in whom the pulmonary veins emptied into the right atrium, showed an almost complete absence of the interatrial septum so that in effect there was but a single atrium. In this, mixing of aerated and nonaerated blood could take place, and the left ventricle would receive an adequate amount of blood, even though this blood might have been inadequately oxygenated. With respect to the third (Kera's case, reported in the Japanese literature), only a brief German summary is available. However, it should be pointed out that the foramen ovale was widely patent, the ductus arteriosus was incompletely obliterated and the patient was a chronic invalid.

In table 2 are collected all the cases of complete drainage of the pulmonary blood into the right atrium or its tributaries. There are 23 cases (table 2 *A*) in which there were no associated cardiac anomalies of importance, particularly none which shunt any of the systemic blood into the left atrium (e. g., see table 2 *B*, cases of Bochdalek, Arnold and Miura). Analysis of these 23 cases shows that the sites of anomalous entrance of the pulmonary veins, in order of frequency, are the superior vena cava and its tributaries (8 cases), then the coronary sinus (7 cases), the right atrium itself (3 cases), the portal vein (2 cases) and the inferior vena cava (1 case). In 1 case the drainage was partly into the right atrium, with the remainder into the superior vena cava; in 1 case, that of a fetus between the fifth and sixth months of development, the pulmonary vein connected with a duct of Cuvier. Of the 23 patients, 12 were males and 9 were females; the sex of 2 others was not stated. In all but 3 (including the 2 fetuses) the foramen ovale was open. In the 3, its condition was not stated. Of the 18 patients in whom the state of the ductus arteriosus could be ascertained, the ductus was patent in one half and closed in the other. In 13 of the 23 cases it was stated that the left side of the heart was disproportionately small.

Of the patients who had associated major cardiac anomalies (table 2 *B*), in 9 the pulmonary veins drained directly into the right atrium or the superior vena cava or the latter's tributaries. In 1, drainage was into the portal vein, and in 2, partly into the portal and partly into the superior vena cava or a branch. In 1 patient the drainage was into

a persistent sinus venosus. As far as could be determined, the anomalies were about evenly distributed between the two sexes.

Several of the cases in which associated cardiac anomalies were observed deserve special mention. Ramsbotham's case, which is frequently cited and usually with incorrect literature reference, was that of a newborn infant with cor biloculare, in which the left pulmonary vein drained into the left subclavian vein, while the vessels from the right lung formed a single trunk which emptied into the portal vein. Bochdalek's case was very similar, except that the heart had four chambers. It is not included with the cases in table *2 A* because the orifice of the inferior vena cava was so situated that it delivered blood partly into each of the atriums. Were it not for that, the left side of the heart would have received no blood, since the ductus arteriosus and the foramen ovale were both closed. The case described by Miura was one in which the right atrium received the blood from the lungs, but the left atrium received the superior vena cava. This, together with the patency of the ductus arteriosus, assured an adequate volume of blood to the major circulation.

Two new cases are added in this report. Both the patients were infants dying at 10 weeks of age. In the first, the pulmonary veins drained into the coronary sinus, then into the right atrium. In the second, the pulmonary veins delivered their blood into the superior vena cava.

## REPORT OF CASES

Case 1.—The infant, a white girl, weighed 7 pounds 5 ounces (3,317 Gm.) at birth. The delivery was uneventful. Cyanosis was not noted and the respirations were normal at birth. Physiologic jaundice was present on the fourth day. The infant was discharged on the tenth day without any abnormalities having been noted.

She did not gain weight. After 4 weeks of age, vomiting frequently followed feedings. At about 9 weeks the vomiting became projectile. At 10 weeks she was brought to the hospital.

The temperature was 97.8 F.; the pulse rate, 130; the respiratory rate, 34. The infant appeared pale, malnourished and mildly dehydrated, weighing 7 pounds 10 ounces (3,458.5 Gm.). She was cyanotic only when crying or active. The heart was overactive and slightly enlarged to the left. A thrill, the position of which in the cardiac cycle was difficult to determine, was present. A rough systolic murmur was heard over the whole precordium, loudest at the apex and the tricuspid area, and also a questionable diastolic murmur. The lungs were clear.

On the morning after admission, the temperature rose to 104.6 F., the child became cyanotic, the respirations were rapid and labored and the pulse rate was over 160. The lungs remained clear. In two hours the child died. The clinical diagnosis was congenital heart disease and cardiac failure.

*Autopsy* (three hours post mortem).—The body was that of a well developed white girl, 57 cm. long. There were no external anomalies. No appreciable cyanosis or edema was present. The precordium did not bulge. The abdominal situs was normal. The distended pericardial sac, containing about 15 cc. of clear

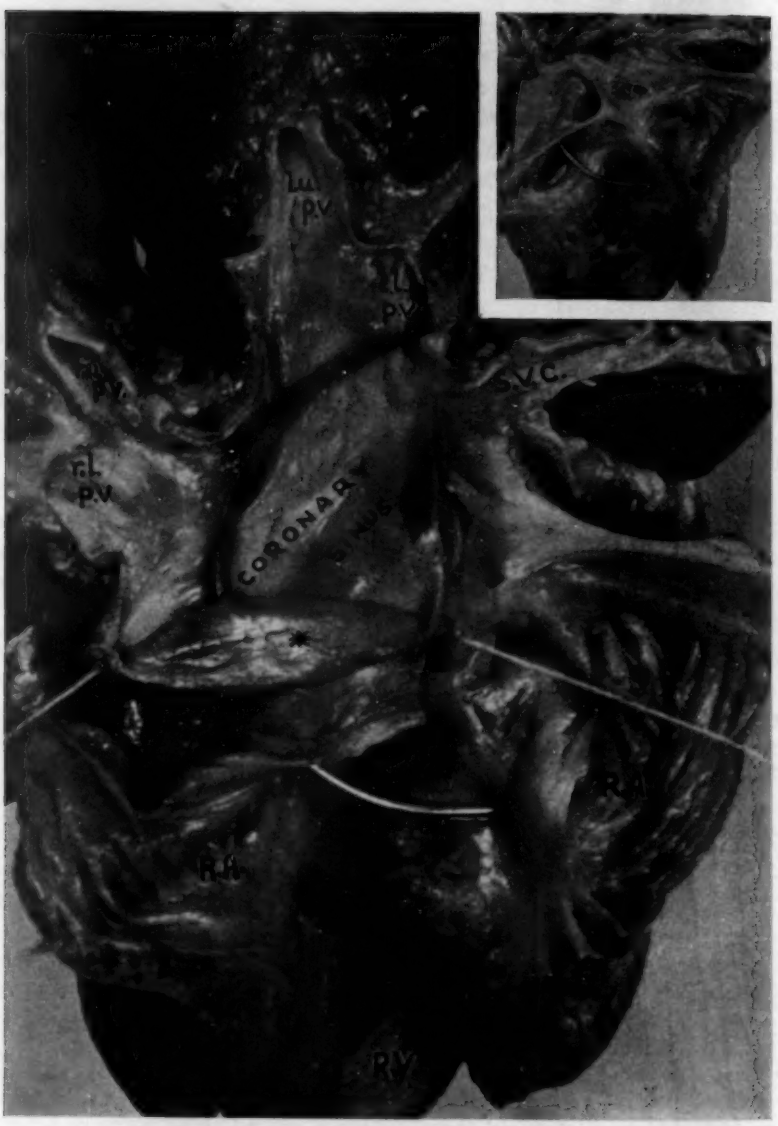


Figure 1

*(See legend on opposite page)*

fluid, occupied more than half of the transverse diameter of the chest. The heart was tremendously enlarged; its transverse diameter in situ was 7 cm. The greatest oblique diameter was 8.5 cm.

The entire anterior aspect of the heart was formed by the dilated right atrium and ventricle. The interventricular sulcus was not readily discernible. The enlarged pulmonary arch obscured the aorta. The great vessels were explored in situ; the ductus arteriosus was present as a cord in the usual position. The vessels of the aortic arch showed the normal arrangement. The right and left pulmonary arteries, each 2 cm. in circumference, followed the normal course but were larger than normal. The pulmonary veins, instead of entering the left atrium, emptied into a large chamber on the posterior aspect of the heart, somewhat posteriorly and to the left of the greatly distended right atrium, and between it and the relatively small left atrium. The chamber was found, after the heart was opened, to communicate with the right atrium by an oval orifice 12 by 10 mm. in diameter immediately below and slightly medial to the normally situated opening of the inferior vena cava. It also received the numerous small coronary veins and was obviously a greatly distended coronary sinus. Chiari's network was found to the right of the opening of the sinus and inferior to the opening of the inferior vena cava. In the normal situation was the foramen ovale, partly closed by the septum primum, leaving only a slitlike orifice, 1.2 by 0.3 cm. It communicated by a funnel-shaped passage with the very small left atrium.

The tricuspid valve was normal, leading into the greatly enlarged right ventricle, whose wall measured 10 mm. in thickness. The pulmonic valve was normal, the ring large. The pulmonary artery immediately above the valve measured 30 mm. in circumference. The left atrium was unusually small, and the only entrance to it was through the imperfectly closed foramen ovale described. The mitral valve was normally formed but small. On its atrial surface were three small "blood cysts." The left ventricle was small, its wall having an average thickness of 5 mm. The interventricular septum was intact. The aortic valve and aortic orifice were normally formed, though small. The coronary orifices were situated at their usual sites. The aorta measured 18 mm. in circumference in its ascending portion, and

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EXPLANATION OF FIGURE 1

Fig. 1 (case 1).—Opened coronary sinus, showing entrances of the four pulmonary veins. A curved needle passes from the sinus directly into the right atrium. The wall of the right atrium (marked with an asterisk) is turned down to show the distended coronary sinus. The left lung is rotated superiorly and medially to bring it into view. The superior vena cava is shifted to the left from behind the coronary sinus. The insert shows the needle coming through the wide orifice of the coronary sinus. Immediately above is the fossa ovalis. A key to the abbreviations in figures 1 and 2 follows:

*r.u.p.v.*, right upper pulmonary vein
*r.l.p.v.*, right lower pulmonary vein
*l.u.p.v.*, left upper pulmonary vein
*l.l.p.v.*, left lower pulmonary vein
*p.v.*, pulmonary vein
*o.p.v.*, opening of common pulmonary vein into superior vena cava
*s.v.c.*, superior vena cava
*R.A.*, right atrium
*R.A.app.*, right auricular appendage
*F.O.*, foramen ovale
*T.V.*, tricuspid valve
*R.V.*, right ventricle

12 mm. in its descending portion, narrowing gradually. The ductus arteriosus was completely occluded; its aortic and pulmonic ends were marked by tiny dimples.

The liver and the spleen were moderately congested. The mesentery and the round ligament were edematous. The ductus venosus and the umbilical vein did not admit probes. There was slight hypertrophy of the musculature of the pylorus but no pyloric obstruction. The remainder of the autopsy revealed no abnormalities. Study of microscopic sections added nothing further.

The anatomic diagnosis in this case was: congenital heart disease; anomalous emptying of the pulmonary veins into the coronary sinus; enlargement of the right atrium and ventricle and of the pulmonary artery; hypoplasia of the left atrium and ventricle; passive congestion of the liver and the spleen; slight hypertrophy of the pyloric musculature.

Case 2.—The infant, a white boy weighing 7 pounds 5 ounces, was born at St. Vincent's Hospital. It was a spontaneous delivery at full term. Immediately after birth slight cyanosis of the face and extremities was noted. This was intermittently present until the day preceding discharge from the hospital, on the twenty-third day. Physical examination was negative except for a rumble heard over the precordium throughout the cardiac cycle. A roentgenogram of the chest revealed slight enlargement of the heart. The hemoglobin was 136 per cent; the red blood cells numbered 5,090,000 and the white blood cells 26,500. The diagnosis on discharge was congenital heart disease.

The infant gained weight well but continued to be cyanotic. On admission to Beth Israel Hospital at 10 weeks of age, he weighed 11 pounds 8 ounces. While he was at rest, moderate cyanosis of lips and nail beds was present. The cry was hoarse. There was moderate respiratory difficulty. The heart was overactive and the beat rapid with neither thrills nor bruit. A loud murmur, difficult to place in the cardiac cycle, was heard. The spleen was palpable. Peripheral edema was not present. A roentgenogram revealed an anomalous cardiac contour, characterized by marked prominence of the conus and increased convexity of the base of the heart, suggestive of right ventricular enlargement. The hemoglobin was 105 per cent, the red blood cell count 5,700,000 and the white blood cell count 9,000.

The day after admission, cyanosis suddenly increased, respirations failed, and there was no response to oxygen or stimulants.

*Autopsy.*—The pertinent findings follow: The body was well developed and nourished. There were no external abnormalities other than cyanosis of the nail beds. The liver weighed 160 Gm. and was engorged with blood. Its edge was 2 fingerbreadths below the costal border. The spleen weighed 12.5 Gm. and was congested.

The pericardial cavity was distended, containing a few cubic centimeters of clear fluid. The heart was markedly enlarged; its transverse diameter in situ was approximately 7.5 Gm. The entire anterior aspect was formed by the right atrium and ventricle. The region of the conus was bulging. The right atrium was distended and its wall hypertrophied. The inferior vena cava entered at the usual site. No remnant of a eustachian valve was found. Immediately below the mouth of the vein a muscular ridge was present, separating it from the small opening of the coronary sinus, which was guarded by an unusually well developed membranous valve. Superior to and continuous with the left wall of the orifice of the inferior vena cava was the fossa ovalis. In the fossa was a narrow oval slit, 6 mm. high and 3 mm. across at its widest point. The anterior wall of the

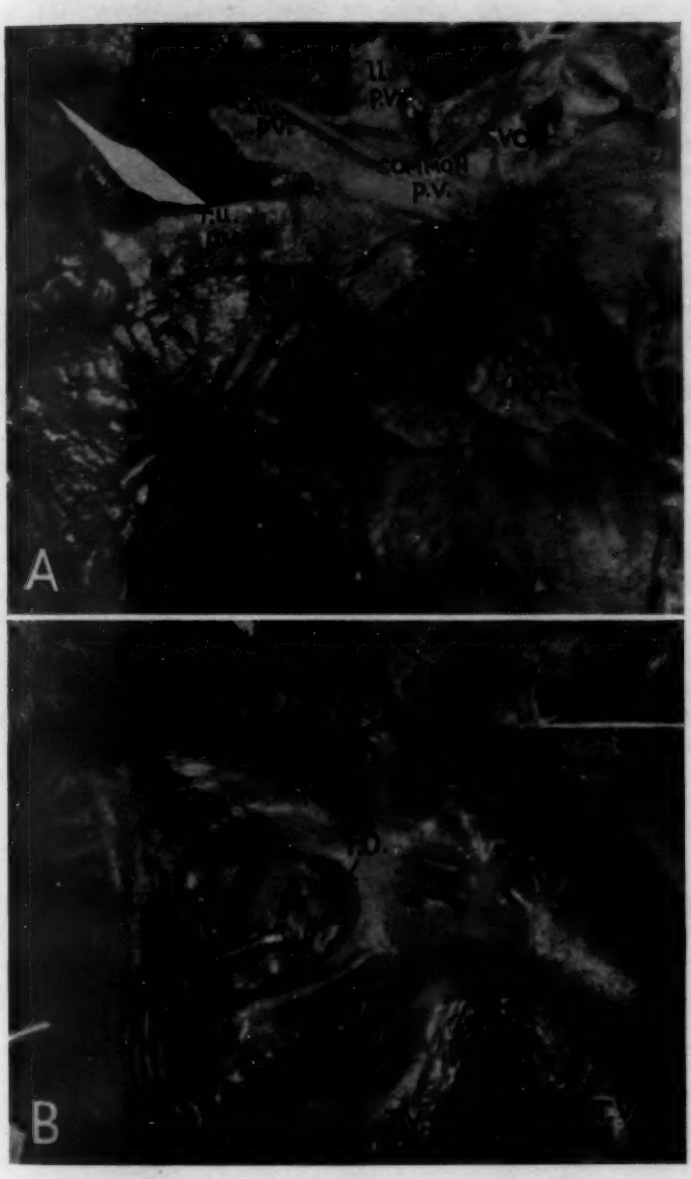


Fig. 2 (case 2).—Pulmonary veins emptying into the superior vena cava. *A*, four pulmonary veins forming a common trunk, which empties into the superior vena cava immediately above the latter's entrance into the right atrium. *B*, opening of the common pulmonary vein into the superior vena cava. A probe passes through the mouth of the inferior vena cava, pointing toward the slitlike foramen ovale.

fossa ovalis was formed by a semicircular muscular ridge, 5 mm. in thickness, which continued superiorly to lie between the fossa and the opening of the superior vena cava, in part forming the latter's inferior border. The diameter of the orifice of this vessel was 5 cm. The tricuspid ring measured 5 cm. in circumference; the valve was normally formed. The right ventricle was dilated, its wall hypertrophied. The trabeculae carneae were prominent. The myocardium varied from 3 to 5 mm. in thickness. The pulmonic valve appeared normal; the ring measured 3 cm. in circumference. The pulmonary artery immediately above the ring measured 3 cm. One and one-half centimeters above its origin, it bifurcated in the usual manner. Six millimeters above the point of bifurcation was the origin of the ductus arteriosus. This was a firm cord, 1.2 cm. long and 4 mm. in diameter. At the pulmonic end a tiny dimple, approximately 0.3 mm. in diameter, was found. A fine probe was forced through this opening and passed out at the aortic end. A small amount of postmortem clot was pushed ahead of the probe tip.

The left atrium was small, and the only entrance into it was the aforementioned slit in the fossa ovalis. The foramen ovale from this side appeared as a circular opening, 5 mm. in diameter. Its posterior edge was formed by the remnants of septum I, and through the opening was seen the inferior portion of septum II. No markings suggested entrances of pulmonary veins. The mitral valve was normally formed but small. Its ring measured 4 cm. in circumference. Except for its small size, the left ventricle was normally formed. Its wall reached a thickness of 3.5 mm. The interventricular septum was intact. The aortic valve was normally formed and intact. The coronary orifices were normally situated. The aorta in its ascending portion measured 1.7 cm. in circumference.

Arising from the right lung were two pulmonary veins. The superior in its extrapulmonary portion was 1 cm. long and 1 cm. in circumference; the inferior was 1.2 cm. long and 1.4 cm. in circumference. They joined in a sinus-like structure and in turn were joined by similar vessels arising from the left lung. The left upper vessel was 2 cm. long; the lower, 1.5 cm. They both measured 12 mm. in circumference. From the sinus-like structure, a short trunk, 6 mm. long and 14 mm. in circumference, led into the superior vena cava through the latter's posterior wall immediately above the entrance of the vein into the right atrium. The superior vena cava was 2.5 cm. long and received the innominate veins in normal fashion.

The anatomic diagnosis in case 2 was: congenital heart disease; drainage of the pulmonary veins into the superior vena cava; hypertrophy and dilatation of the right atrium and ventricle; hypoplasia of the left atrium and ventricle; passive congestion of the liver and the spleen; slight interstitial emphysema of the lungs.

## COMMENT

Brown showed, in the cat, that the intrapulmonic portion of the pulmonary circulation arises from part of the presplanchnic plexus. Flint, from studies of the fetal pig, and Auer, from studies of the rat and rabbit, held that the main pulmonic stem arises from the sinus venosus. Communication of this stem with the vessels of the presplanchnic plexus in the chick was described by Buell and more recently by Chang. Most embryologists who have studied the problem (Flint; Brown; Bremer;

Davies and MacConaill) have placed the initial position of the opening of the common pulmonary stem in the dorsal wall of the sinus venosus as central. With the formation of the interatrial septums, this communication shifts to the left.

Davies and MacConaill have brought forward an attractive anatomical explanation for this shift of the pulmonary vein. They have supposed an active part played by the "pulmonary fold," an invagination of the right wall of the common pulmonary trunk into the sinus venosus, in the separation of the right and left parts of the primitive atrium. They present a case in which the incorporation of the stem of the pulmonary vein into the wall of the sinus venosus is independent of the process of formation of that essential element of the complete atrial septum which they call the "pulmonary fold." In their case the atrial septums were completely absent.

In the 2 cases described here the atrial septums were present, as they were in all the cases listed in table 2 *A*. In all the cases listed in this table except those of Kera, Engels and Duff, the pulmonary veins maintained no direct connection with the atriums. There is no reason to assume that there ever had been such communication.

In the cases of Kera, Engels and Duff, as well as those of Rokitansky, Audry and Lacroix, and Wood and Williams, as Davies and MacConaill suggested, the opening of the pulmonary veins into the right atrium may possibly be due to the failure of formation of the "pulmonary fold." Bremer, in his discussion of the factors involved in the formation of the heart chambers and the great vessels, postulated that in the anomalies in which the pulmonary vein enters the vena cava "the median septum closed to the left of the vein mouth, showing that here nature is delicately balanced." He suggested further that "in the chick at least, the (pulmonary) vein and the left valve of the sinus become separated by new cells developing between, that each thus moves, the vein to the left slightly, the valve further to the right, and that the septum then grows down in the midline, just to the right of the vein, but some distance away from the valve, thus forming the spatium interseptovalvulare described by Tandler in the human heart." The formation of the septum primum, then, to the left of the pulmonary vein, or the failure of formation of the "pulmonary fold" of Davies and MacConaill might account for the incorporation of the mouth of the pulmonary vein in the right atrium or the adjacent vena cava. A somewhat similar explanation would account for the communication with the coronary sinus. This structure arises from the central portion and left lateral horn of the sinus venosus. Thus, if the pulmonary trunk maintains its connection with the transverse portion of the sinus venosus, it will drain through this into the right atrium, with which chamber this structure eventually communicates. In the first case presented here, and in the cases of Nabarro,

Nagel, Terplan and Sanes and Palmer (his case 2), the aberration in development was a little later, or rather the development had progressed to a slightly later stage, than in the cases of DeGroat and Thatcher and Palmer (his case 1). In the latter, the pulmonary veins joined to form a common trunk before emptying into the coronary sinus. In the former 5 cases the subsequent separation of the right and left pulmonary veins occurred as normally. This explanation of the communication of the pulmonary veins with the coronary sinus was originally suggested by Nabarro in 1902, who described the first such case to be reported, and is given briefly by Maude Abbott in her "Atlas of Congenital Cardiac Disease."

When the pulmonary veins communicate with more distant tributaries of the right atrium (innominate veins; portal vein), these explanations will not suffice. It seems necessary to fall back on the retention of the one or the other of the original intercommunications of the presplanchnic plexus. That the retention of such primitive communications is not exceedingly rare is borne witness to by the cases in table 1. These may be physiologically unimportant, as the age of many of the patients attests. The cases in table 2 represent the anatomic extreme of those in table 1. Whether the retention of the splanchnic communications or the lack of communication between the pulmonary veins and the atrium represents the primary defect is problematic. Chang evidenced a belief that the failure of primary communication with the heart would lead to free communication between the pulmonary veins and the systemic veins. The occurrence of isolated communications between the pulmonary and the major circulation in which the greater portion of the pulmonary drainage is into the left atrium shows that the two processes are not mutually exclusive. However, it may be that any pathway that permits emptying of the blood from the pulmonary bed suffices to answer the physiologic demands in the developing embryo. The normal pathway represents the usual anatomic pattern. Any interference with the normal pattern will procure the retention of some other outlet for the pulmonary current. Any of the numerous splanchnic anastomoses will answer.

The dynamics of circulation in these hearts are interesting. In all cases in which the information is available (see table 2 *A*), the foramen ovale was open. In about one half, the ductus arteriosus was closed or almost so. In the latter instances all the blood reaching the systemic circulation passed through the aortic valve and, to reach the left side of the heart, had to pass through the foramen ovale. This blood was a mixture of aerated and nonaerated blood, for all the oxygenated blood returning from the lungs was mixed eventually in the right atrium with blood returning from the major circulation. In the cases of Nagel and Terplan and Sanes, which concerned 84 day and 5 month old infants,

respectively, in which the foramen ovale was slitlike and in which the ductus arteriosus was closed, the choking off of the interatrial communication and thus of the blood supply of the greater circulation may well have been the cause of death. It is interesting to note that in all patients of this series (table 2 *A*) older than 3 months the foramen ovale was open, whereas in all but 2 the ductus arteriosus was closed. Patten stated that the ductus arteriosus is normally completely closed between the sixth and eighth week and that the foramen ovale is reduced to probe patency by the first month, with complete fibrous occlusion occurring by the last third of the first year. However, in from 20 to 25 per cent of all persons anatomic patency may remain through life. Examination of the reports of all cases in which any part of the pulmonic return is to the right side of the heart reveals, when information is available, that in much more than half the foramen ovale remained patent beyond the first year of life. It is tempting to argue that this represents an attempt to compensate for an imbalance between the right and the left chambers of the heart. However the number of cases in which data are available is small, and the figures are probably weighted by the fact that the normally closed foramen is probably not mentioned whereas the abnormally open one is.

Of all the patients in whom the entire pulmonary drainage was into the right side of the heart, whether complicated by other cardiac anomalies or not, only 3 lived beyond early childhood. The patient whose case was reported by Wood and Williams was a 15 year old girl, who died of meningitis and who had suffered from chronic cardiac disease all her life. She had shown dyspnea on exertion and clubbing of the fingers. Autopsy revealed true cor biloculare. The only reason this case is included here is because the pulmonary veins entered the right side of the single-atrial cavity. As the authors stated in their discussion, numbers of persons with cor biloculare have survived into the second decade of life. The blood supply to the greater circuit is adequate in amount even if it is inadequately oxygenated. The 19 year old youth described by Rokitansky and the 27 year old woman described by Kera have already been alluded to. The former was a patient with essentially a single-atrial heart, so that from the purely dynamic standpoint the left ventricle received an adequate supply of blood. An adequate description of Kera's case is not available. Adachi's brief summary makes it clear that the woman was chronically ill, presumably with a cardiac embarrassment. At autopsy the heart showed a foramen ovale open to the width of admitting a "thumb" and a ductus arteriosus having a lumen 1 mm. in diameter. The balance between the right and the left side of the heart had to be maintained through the foramen ovale and the ductus; the gradual closing of the latter might have produced imbalance, with decompensation and death.

There are 25 cases (table 1) in which 75 per cent or more of the return (assuming each of the major pulmonary veins carried an equal load) from the lungs was to the left atrium. All but two of the patients lived to be adults. One of these 2 patients was a 133 day old infant with a heart showing complicated anomalies; the other was a 12 year old child dying of pneumonia and pulmonary abscess. There were 2 cases in which approximately 60 per cent of the return was to the left side of the heart, and both the patients were adults. In 15 cases in which the age was known, about 50 per cent of the venous return from the lungs was normally received in the left side of the heart. Seven were adults. Of the 8 children, all but 1 were less than 1 year of age. It seems, therefore, that in the absence of some counteracting factor (e. g., a large atrial septal defect) about 50 per cent return to the right atrium is the boundary line which determines whether compensation will be possible. When less than 50 per cent is abnormally carried into the major venous circuit, there is little likelihood of decompensation, and the patient reaches an adult age.

In all of the cases in table 2 *A* the hearts were virtually fetal in type, for the right atrium received both oxygenated and partially deoxygenated blood (see papers of Kellogg and Windle and Becker for discussions of the mixture or the separation of the venous streams as they pass through the right atrium of the fetus). Their circulation was evidently adequate for normal growth and the lower levels of activity characteristic of the fetal and neonatal periods. However, cyanosis, particularly during activity, characterized most of the patients.

With increasing demands made by increasing muscular activity, these hearts became inadequate. In most of the cases it is noted that not alone was the right side of the heart hypertrophied but the left side of the heart was smaller than normal. So long as the ductus arteriosus remained open, the right ventricle could supply the greater part of the major circulation. However, except in the case of Kera, as mentioned in an earlier paragraph, only in 1 infant living longer than 3 months (Miura's patient, 6 months) was the ductus arteriosus still patent, and interestingly enough in this infant the left atrium received blood from the superior vena cava. As early as the tenth week of life the ductus arteriosus closed, in the face of a need for its remaining open. The closing of the ductus arteriosus would have two effects: first, the blood supply to the major circulation would of necessity be reduced; second, the load on the right side of the heart would be increased. The return from both circuits is to the right atrium, while the outflow is reduced by the amount formerly passing through the ductus. This must lead to dilatation of the right side of the heart, hypertrophy and eventually failure.

In the 2 cases reported here, and in the cases of Ghon, Nagel, and Terplan and Sanes and perhaps a case of Palmer's the evidence suggests that the foramen ovale was also closing. It seems probable that when the amount of blood flowing through the narrowing foramen ovale became insufficient to supply the demands of the systemic circulation, and, pari passu, the right side of the heart received a greater supply of blood than it could handle, decompensation ensued.

As was mentioned by Nagel, these cases add to the doubts which have been cast on the usual mechanical, physiologic and teleologic explanations of the closure of the ductus arteriosus. It may be necessary to fall back on as yet unknown ontogenetic factors to explain the closure of this shunt in the face of adverse conditions and with fatal effect.

## SUMMARY

Drainage of all or of part of the lungs into the major venous system is relatively uncommon. One hundred cases, of which 35 represent total drainage into the right atrium or its tributaries, have been collected from the literature. Two additional cases are described; in one the pulmonary veins drained into the coronary sinus, and in the other, into the superior vena cava. Of the 37 patients with complete drainage into the right side of the heart or its tributaries, only 8 lived beyond 6 months. In all cases in which the information is available, the foramen ovale was open. In about one-half, the ductus arteriosus was closed or almost closed. In the latter, all the blood reaching the systemic circulation passed through the aortic valve and, to reach the left side of the heart, passed through the foramen ovale. As the balance between the right and the left side of the heart in these cases had to be maintained through the foramen ovale and the ductus arteriosus, the gradual closing of these, particularly the latter, might have produced imbalance, with decompensation and death. In comparing cases in which there was partial or total drainage into the right side of the heart, it appears that when less than 50 per cent of the pulmonary return is abnormally carried into the major venous circulation, there is little likelihood of decompensation, and such persons reach adult age.

The possible embryologic explanations of these cases are briefly discussed.

## ADDENDUM

Since this paper has gone to press, we have seen at autopsy 2 instances of pulmonary veins draining into the right side of the heart and found 2 others reported in the literature. Of the two former anomalies, one was in a 56 year old woman, dying from postoperative pulmonary emboli. A smali pulmonary vein from the right lobe joined the azygos vein at the latter's point of entrance into the innominate vein. A valve

was present at the mouth of the pulmonary vein. The second was in a stillborn boy, whose death was due to fracture of a cervical vertebra, tentorial tears and cerebral hemorrhage. A vein draining the upper half of the upper lobe of the right lung emptied into a branch of the superior vena cava. In addition, there was coarctation of the aorta, with dilatation of the pulmonary artery. The right atrium and ventricle were hypertrophied. The aortic valve was bicuspid. Anomalies of the lungs and the urinary tract were also present.

In 1912, Park observed in a 2½ month old boy that the veins from the right lung communicated with the inferior vena cava above the diaphragm.

In 1941, McManus described the circulatory system of a 12 week old boy, in which both pulmonary veins emptied into a persistent left superior vena cava. This communicated with the right superior vena cava, and thus with the right atrium. Both the foramen ovale and the ductus arteriosus were slightly patent. The left auricle and ventricle were very small.

These cases increase to 38 the number with total drainage into the right atrium or its tributaries, and to 68 those with incomplete drainage.

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# PATHOLOGIC ASPECT OF NUTRITIONAL DEFICIENCIES IN RATS

## I. LESIONS PRODUCED BY DIETS FREE OF VITAMIN $B_6$ (PYRIDOXINE) AND THE RESPONSE TO VITAMIN $B_6$

WILLIAM ANTOPOL, M.D.
NEWARK, N. J.
AND
KLAUS UNNA, M.D.
RAHWAY, N. J.

Vitamin $B_6$ deficiency was first described by György.[1] In nutritional studies on rats, he showed that a characteristic dermatitis, for which he coined the name "rat acrodynia," was caused by the lack of a hitherto unidentified factor of the vitamin B complex, termed vitamin $B_6$. As a chemical entity—2-methyl-3-hydroxy-4,5 bis-(hydroxymethyl)-pyridine—vitamin $B_6$ (pyridoxine) was isolated from rice bran and yeast in 1938 almost simultaneously by Keresztesy and Stevens,[2] Lepkovsky,[3] György [4] and Kuhn and Wendt.[5] Synthesis of vitamin $B_6$ was accomplished in 1939 by Harris and Folkers [6] and by Kuhn and co-workers.[7]

Biologic studies on vitamin $B_6$ have been largely confined to the rat. However, the necessity of vitamin $B_6$ and its role in nutrition have been demonstrated in other species. Dogs maintained on a diet free from vitamin $B_6$ show microcytic hypochromic anemia, which does not respond to iron but which is readily cured by vitamin $B_6$ (Fouts and co-workers [8]; McKibbon and co-workers [9]). Vitamin $B_6$ deficiency has

From the Division of Laboratories, Beth Israel Hospital, Newark, N. J., and the Merck Institute for Therapeutic Research, Rahway, N. J.

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8. Fouts, P. J.; Helmer, O. M.; Lepkovsky, S., and Jukes, T. H.: J. Nutrition **16**:197, 1938. Fouts, P. J.; Helmer, O. M., and Lepkovsky, S.: Proc. Soc. Exper. Biol. & Med. **40**:4, 1939; Am. J. M. Sc. **199**:163, 1940.
9. McKibbon, J. M.; Madden, R. J.; Black, S., and Elvehjem, C. A.: Am. J. Physiol. **128**:102, 1939.

been produced in the chick (Jukes[10]), and the important role of this vitamin has also been shown in the nutrition of pigs (Chick and co-workers[11]; Wintrobe[12]). The need for vitamin $B_6$ in man has not been clearly demonstrated.

In the course of studies on the requirement of rats for vitamin $B_6$ and other factors of the vitamin B complex, it was decided to investigate the pathologic and histologic aspects of the deficiency syndrome occurring with diets free from vitamin $B_6$, to observe the effects of the vitamins on this syndrome[13] and to compare these findings with those for control animals maintained on the same basal diets to which an adequate supply of vitamin $B_6$ had been added. By these studies it was hoped that a differentiation between vitamin $B_6$ deficiency and other deficiencies

*Composition of Diets SS, SD, D and 235*

| | SS | SD | D | 235 |
|---|---|---|---|---|
| Vitamin-free casein (Labco) | 18% | 18% | 18% | 18% |
| Corn starch | 68% | 34% | .... | .... |
| Dextrose (cerelose) | .... | 34% | 68% | 68% |
| Hydrogenated vegetable fat (crisco) | 8% | .... | 8% | .... |
| Butter fat | .... | 8% | .... | 8% |
| Salt mixture (U. S. P. XI, no. 1) | 4% | 4% | 4% | 4% |
| Cod liver oil | 2% | 2% | 2% | 2% |
| Thiamine, mg. per 100 Gm. of diet | 0.8 | 0.8 | 0.8 | 0.8 |
| Riboflavin, mg. per 100 Gm. of diet | 0.8 | 0.8 | 0.8 | 0.8 |
| Nicotinic acid, mg. per 100 Gm. of diet | (10)* | (10)* | .... | 10.0 |
| Choline chloride, mg. per 100 Gm. of diet | .... | 100.0 | .... | 100.0 |
| Calcium pantothenate, mg. per 100 Gm. of diet | .... | .... | .... | 5.0 |
| Liver concentrate free from vitamin $B_6$, cc. per 100 Gm. of diet containing 3.69 mg. of pantothenic acid per cubic centimeter | 0.6* | | | |

* This substance was added only in a special series of experiments.

of the vitamin B complex could be made. Similar studies with pantothenic acid are in progress.

## METHOD

Twenty-one day old male albino rats of a Wistar strain were placed in individual cages in an air-conditioned room. Water and food were supplied ad libitum. The composition of the diets used in this study is shown in the table.

Diet SS resembles closely the diet used by György[14] in his early work with the exception that rice starch was replaced by corn starch. In other diets (SD, D, 235) dextrose was substituted for corn starch, a change which did not influence

10. Jukes, T. H.: Proc. Soc. Exper. Biol. & Med. **42**:180, 1939.

11. Chick, H.; Macrae, T. F.; Martin, H. A., and Martin, C. J.: Biochem. J. **32**:2207, 1938.

12. Wintrobe, M. M.: Am. J. Physiol. **126**:325, 1939.

13. Antopol, W., and Unna, K.: Proc. Soc. Exper. Biol. & Med. **42**:126, 1939.

14. György, P.: Biochem. J. **29**:741, 1935.

the character of the lesions. However, the appearance of the deficiency lesions was markedly accelerated when corn starch was replaced in part (diet SD) or entirely (diets D and 235) by dextrose (Sampson and Unna[15]). No effect on the deficiency lesions was observed following the substitution of the vegetable fat (diet SS) by butter fat (diet SD and 235).

Vitamins A and D were supplied in cod liver oil. Thiamine and riboflavin were added to all rations in adequate amounts. Nicotinic acid, apparently not needed by the rat, was supplied to only part of the animals, but no effect on the deficiency lesions was observed following the addition of this substance. Choline was included in diets SD and 235. Diet SS was fed to the majority of the animals used for histologic studies. In a series of experiments this diet was supplemented with a liver concentrate which contained pantothenic acid and possibly as yet unidentified nutritional factors, but which was free of vitamin $B_6$. Pantothenic acid, the newest member of the vitamin B complex, which became available in pure form only after this study had been started, was included in diet 235.

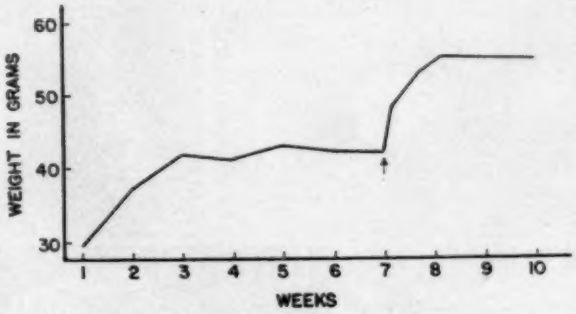


Fig. 1.—Growth of 21 day old male rats maintained on diet SD. The arrow indicates the time at which 50 micrograms of pyridoxine (vitamin $B_6$) was fed to each rat in a single dose.

## GENERAL OBSERVATIONS

The young rats continued to grow on these diets for three to four weeks; then their weights became stationary at 40 to 65 Gm. (fig. 1). The rats on diets SS and 235 reached somewhat higher weights than those on diets SD and D. About the time when the rats ceased to grow, the first signs of dermatitis became noticeable, the lesions observed being quite similar to those first described by György.[14] The paws showed hyperkeratosis, which was rapidly followed by pronounced congestion and edema, with loss of hair. The process usually started on the front paws but reached the most severe stages on the hind paws (fig. 2 *A*). The edema and the concomitant cutaneous lesions became so extreme that in some cases the skin ridges were not discernible (fig. 2 *B*), and even localized hemorrhage was noted. The lesions were most evident on the dorsal surface. At times, hemorrhage, ulceration and necrosis

15. Sampson, W. L., and Unna, K.: To be published.

of the terminal phalanges were present, and the rats tended to eat the affected parts, thus producing amputation, usually at the joints.

Simultaneously or shortly after the beginning of the hyperkeratosis on the paws, the ears and the snout became involved. At first the ears became edematous and congested, later markedly thickened, scaly and distorted. In extreme stages the thickening reached such a degree as to give the ears a cauliflower-like appearance (fig. 2 *D*). The snout became swollen (fig. 2 *C* and 2 *D*), the edema progressing rapidly toward the mouth. Loss of hair kept pace with this change, and later ulceration was noticeable. Secondary infection, increased nasal secretion and

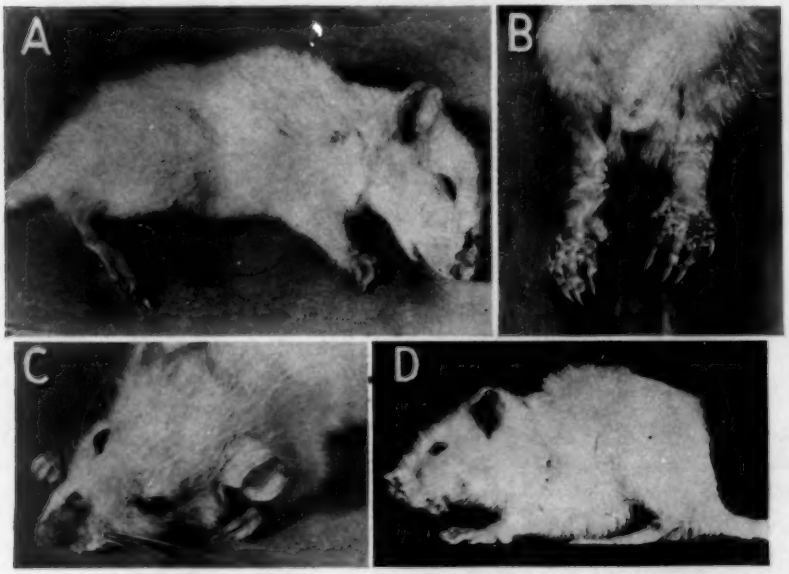


Fig. 2.—Various manifestations in rats on SS diet: *A*, extensive edema of the paws, more pronounced on the hind ones. Note hemorrhage of a terminal phalange of a hind paw. *B*, alopecia with extensive edema and ulceration of paws. Note priapism with scaling at the tip of penis. *C*, alopecia with increased scaling of the skin at the snout. *D*, thickening and distortion of the ears with loss of hair. The unkempt appearance of the fur is due to a deficiency of pantothenic acid and not to the lack of vitamin $B_6$.

ulcerations of the ventral angle of the tongue were not uncommon. Priapism was noted early, and at times the tip of the penis also showed scaling and keratinization (fig. 2 *B*).

On diets containing no pantothenic acid (SS, SD and D) one of the earliest signs was loss of the long hairs of the cover coat. The fur lost its luster, the animal presented an unkempt appearance (fig. 2 *D*) and later localized areas of alopecia appeared. The vibrissae were often

covered with a red or brownish crust, and the fur, especially that over the neck, sometimes showed rusty spots. On diets containing pantothenic acid, although the lesions of the ears, snout and paws were the same, an unkempt appearance of the fur and alopecia were not produced; the fur remained well groomed and failed to show any striking changes except for possible thinning of the hair at the dorsocephalic and dorsocaudal ends of the trunk. If, however, vitamin $B_6$ was added to a diet free from pantothenic acid, the ear, snout and paw changes did not appear, but the alopecia and discoloration of the vibrissae and the fur still occurred.

The average time necessary to produce severe acrodynial lesions was nine to ten weeks on diet SS and five to eight weeks on diets SD, D and 235. Once the lesions became manifest, the mortality of the animals was rather high, many rats succumbing rapidly to secondary infections.

In order that the effect of the administration of vitamin $B_6$ might be observed, rats in severe stages of the deficiency were given the synthetic vitamin by stomach tube either in a single dose or by daily feeding. The rats were killed and examined at intervals varying from twelve hours to four weeks after the start of the administration of the vitamin. At times one ear of the deficient animal was removed to serve as a comparison for the signs of healing observed on the other ear following the administration of vitamin $B_6$.

A single dose of 50 or 100 micrograms of vitamin $B_6$ produced complete healing of the lesions of the ears, paws and snout within seven to fourteen days, accompanied by increase in weight of approximately 15 Gm. (fig. 1). If no further vitamin $B_6$ was administered, the lesions reappeared within one to three weeks. Daily dosing with 5 or 10 micrograms of the vitamin produced weight gains of from 1 to 1.5 Gm. per day for about two weeks, at which time the lesions were healed. The weight then leveled off, and the lesions did not reappear. On diets SS, SD and D, deficiency lesions attributed to the lack of pantothenic acid [16] became more pronounced in the rats after they received vitamin $B_6$, and most of the animals died within two to four weeks in spite of the dramatic improvement in the acrodynia. On diets containing pantothenic acid the weight responses to vitamin $B_6$ were much more striking, and no deaths were recorded over a period of one month.

In addition to experiments with young rats, adult albino rats of the same strain were placed on diets SD and 235. With these rats, ten to twelve weeks were required to produce cutaneous lesions. However, these lesions were not as severe as those obtained within five to eight weeks in 21 day old rats. The lesions of young black and piebald rats showed no difference in appearance or in character when compared with

16. Unna, K.: J. Nutrition **20**:565, 1940.

those in the albino rats on the same diets except that in the absence of pantothenic acid graying of the fur was a striking manifestation.[17]

### HISTOLOGIC STUDIES

More than 300 rats on the various diets deficient in vitamin $B_6$ were examined histologically.

*Ears.*—The most characteristic changes were those observed in the ears. Normally, the ears are delicate, the cartilage is not irregular and

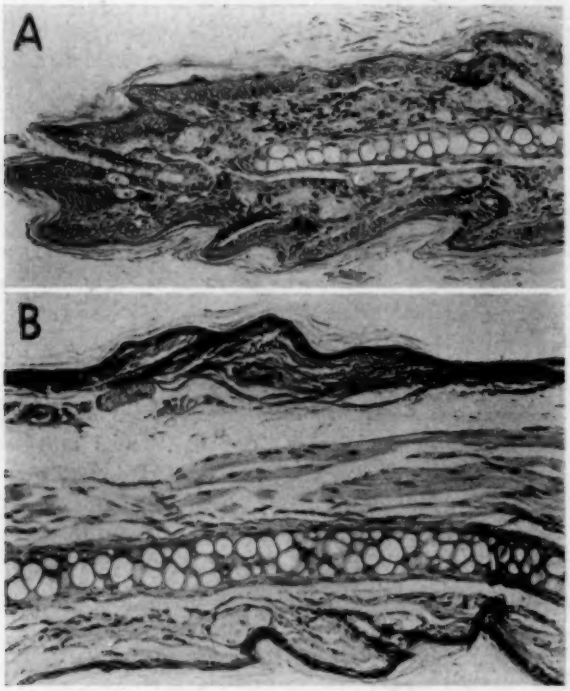


Fig. 3.—Structure of the normal ear: *A*, tip of an ear showing epithelium consisting of one to three layers of cells and little stratum corneum (× 140). *B*, cartilage of an ear of uniform thickness and surrounded by a thin compact layer of perichondrial connective tissues. Note the appearance of normal muscle and sebaceous gland (× 140).

the epithelium consists of from one to three layers of flattened cells (fig. 3 *A*). There is little stratum corneum. The stratum lucidum is thin but discernible. The stratum granulosum is not conspicuous and consists of one layer of cells. Few mitotic figures are seen in the basalis

17. Unna, K., and Sampson, W. L.: Proc. Soc. Exper. Biol. & Med. **45**:309, 1940.

except at the tip of the ears, where they are occasionally encountered. On the anterior portion of the ear the papillary layer is more conspicuous than on the posterior surface, and occasional histiocytes are present in the subcutaneous tissue. The sebaceous glands are prominent, and hair follicles are frequent. The cartilage is fairly uniform in thickness and is surrounded by perichondrial connective tissue, which is intimately associated with it. The muscular attachment to this peri-

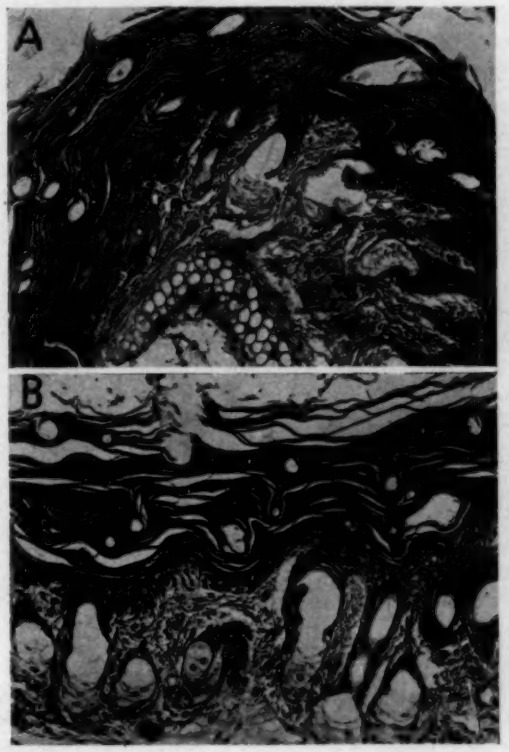


Fig. 4.—Ear of rat on diet SS for seventy-two days: *A*, tip of an ear showing hyperkeratosis, swollen perichondrial connective tissue and prominent stratum granulosum (× 135). *B*, midportion of an ear showing pronounced hyperkeratosis and acanthosis. The pilosebaceous pores are distended (× 140).

chondrial connective tissue extends over a broad area, with little intervening connective tissue (fig. 3 *B*). The muscle fibers at the base of the ear extending on to the back of the head are uniform in size and are compact.

After sixty-five days on diet SS, there was moderate thickening of the perichondrial connective tissue, which was loose in structure. Fat pervaded the muscle bundles, which were somewhat smaller in size and

atrophic in appearance. There was an increase in the number of capillaries in this region. After seventy-two days, the epithelial cells were larger and more vesicular. Hyperkeratosis was pronounced, and degenerating nuclei were seen in the stratum corneum. The cornified zone was often one to one and a half times the thickness of that of the entire epithelial layer. The stratum lucidum merged imperceptibly with, and could not be differentiated from, the stratum corneum. The stratum granulosum was prominent, extending over one to four layers of cells, and the cells, instead of being thin and flat, were swollen and rounded. The prickle cell layer was more developed on the anterior surface of the ear, and intercellular edema was evident. The epithelial pegs dipped

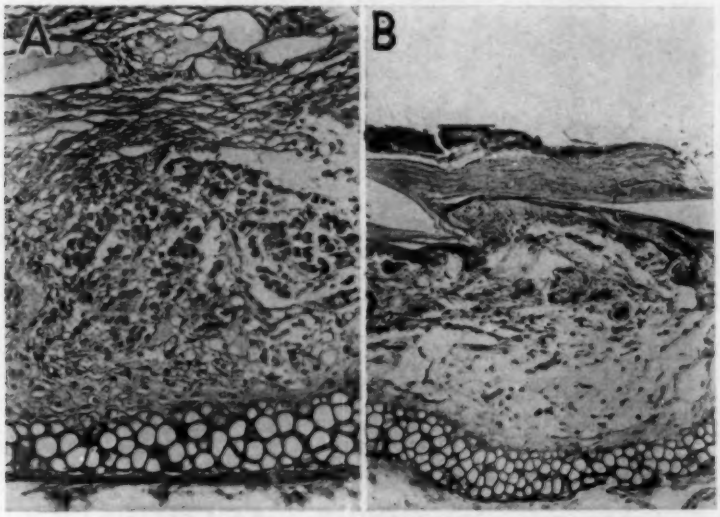


Fig. 5.—*A*, ear of rat on diet SS for seventy-two days, showing atrophic muscle bundles with edematous perichondrial connective tissue at the base of the ear (× 140). *B*, ear of rat on diet SS for seventy-two days, showing edematous and indistinct perichondrial connective tissue and pronounced edema of the subcutaneous connective tissue (× 135).

deeply into the corium. Mitotic figures were not infrequent in the basal layer. The pilosebaceous pores were distended, and often hyperkeratotic material extended into them (fig. 4 *A* and 4 *B*). The associated hair follicles were often atrophic. There was an accumulation of histiocytes and occasional round cells in the corium, which was markedly edematous and hypervascularized. There was marked increase in fat and histiocytes about the cartilage, with marked edema of the perichondrial connective tissue. The muscle bundles had lost a great deal of their striation and appeared degenerated and atrophic (fig. 5 *A*). In seventy-nine days the lesions had progressed to an even greater extent than those of one

week previous. Hyperkeratosis, occasionally with the preservation of nuclei or disintegration of the stratum corneum, as well as acanthosis, was more pronounced. The distended pilosebaceous pores were now atrophic in appearance, and few hair follicles were seen. The perichondrial connective tissue was indistinct and appeared as an edematous, loose fibrous tissue, in places giving the impression that cartilage was

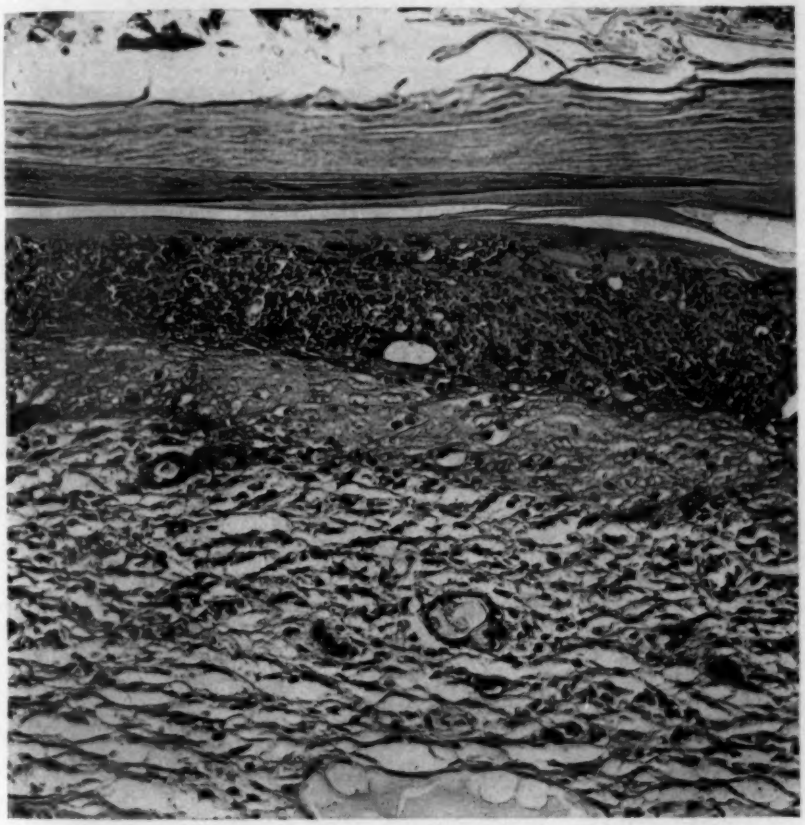

Fig. 6.—Ear of rat on diet SS for seventy-nine days, showing accumulation of fluid, disrupted cells and polymorphonuclear leukocytes between stratum granulosum and corneum, giving the appearance of vesiculation (× 270).

in contact with the surrounding vascularized adipose tissue, which contained histiocytes and round cells (fig. 5 *B*). The muscle fibers showed marked degeneration. The cartilage proper was irregular, distorted and angulated, showing considerable variation in thickness. In places there was lack of continuity between the various segments. The subcutis showed pronounced edema and infiltration by inflammatory cells, including polymorphonuclear leukocytes. Necrosis at the tips of the

ears was not uncommon in the extreme stages, and in these regions there was an abundance of polymorphonuclear leukocytes. In areas, the stratum granulosum was disrupted, and accumulations of fluid or aggregates of polymorphonuclear leukocytes were present between the stratum granulosum and the corium, giving the appearance of vesiculation (fig. 6). Mitotic figures were numerous in the basal layers of the epidermis, and in places the picture was reminiscent of the marked proliferation seen in the region of burns or after the application of tar or other carcinogenic substances.

Within twenty-four hours after the oral administration of 100 micrograms of vitamin $B_6$, the edema receded considerably, and the perichondrial connective tissue was less disorganized. The number of mitotic figures in the basal layers of the epidermis appeared to be further increased in the first twelve hours after treatment (fig. 7), and this was followed by diminution of activity. Sebaceous glands became more evident, and newly formed hair buds appeared in the upper zones of the dermis. After two days, except in the region of the ulcerated areas, the polymorphonuclear leukocytes diminished in number and were replaced by histiocytes and round cells. Mitotic figures were not conspicuous. The sebaceous glands again became prominent and in localized areas hair buds appeared more numerous. The organization continued, so that within one to two weeks there was considerable restitution toward the normal picture. The hyperkeratotic layer at times desquamated in the form of a cast of the ear. A slight amount of edema still remained, the cartilage still showed some distortion, the muscle bundles had only a slight tendency toward normal restitution, and the intermuscular fat tissue diminished somewhat but did not entirely disappear.

With repeated feedings, there was almost complete restitution of the normal picture in two to three weeks. If the dose was not repeated, recurrence was noted in about one to three weeks.

*Abdominal Skin.*—The epithelium of the skin over the abdomen and back is normally one to three layers deep, consisting mostly of flattened cells. The stratum lucidum is quite prominent. The stratum granulosum is one layer thick and distinct (fig. 8*A*). In this region, after seventy-two days on diet SS, the epidermis was usually thinner, and the stratum granulosum was less distinct. The stratum corneum might be somewhat more developed in localized areas. At times the keratosis extended into the hair follicles. The hair follicles were not as numerous and appeared atrophic in many places. The papillary buds were more spongy. There was often edema of the supramuscular connective tissue, as well as degeneration and atrophy of some of the superficial muscular fibers. In seventy-nine days, the edema became even more pronounced and extended into the deeper tissues. The hair follicles appeared

atrophic and in places were scarce. The sebaceous glands were inconspicuous (fig. 8 *B*). The skin of the back showed areas of atrophy with considerable edema in the subcutis. The superficial muscle layers in these regions showed varying degrees of degeneration. Hair follicles and sebaceous glands were infrequent and inconspicuous.

Fig. 7.—Acanthosis with numerous mitotic figures in the basal layers eighteen hours after the administration of 100 micrograms of vitamin $B_6$ to a rat on diet SS for eighty-five days.

Within forty-eight hours after the oral administration of 100 micrograms of vitamin $B_6$, the edema was lessened, and there was an initial period of epithelial activity. Numerous focal groups of new hair buds appeared in the upper portion of the cutis (fig. 9 *A*). Within one week the epithelium appeared normal, with a prominent stratum granulosum.

With continued development, the hair buds were pushed into the deeper regions (fig. 9 *B*), as was pointed out by Wolbach in his studies on the development of the hair follicle.[18] An occasional hair follicle showed collections of polymorphonuclear leukocytes. The atrophic hair follicles, however, did not show a restitution of activity in all places, thus leaving extensive areas of atrophy which sometimes went on to complete bald-

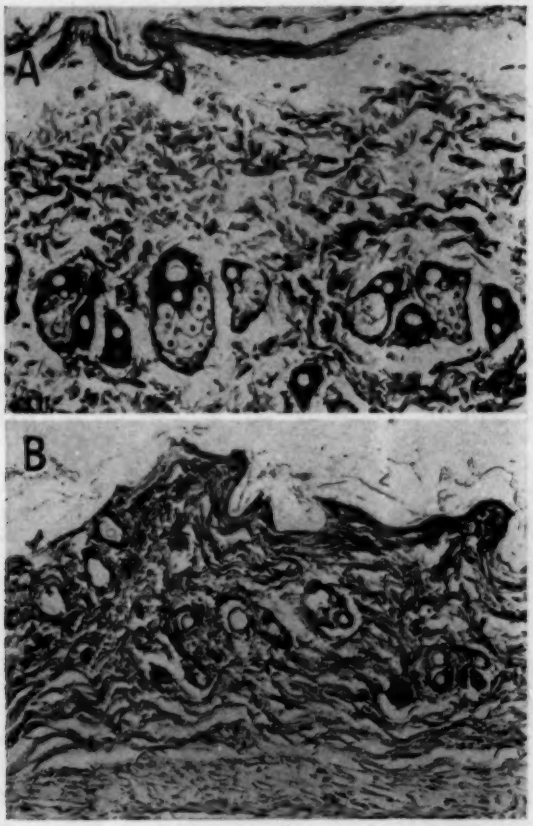


Fig. 8.—*A*, normal abdominal skin with prominent stratum lucidum. The stratum granulosum is one layer thick (× 280). *B*, skin of a rat on diet SS seventy-nine days, showing thin epidermis, atrophy of hair follicles and inconspicuous sebaceous glands (× 280).

ness despite extensive vitamin $B_6$ therapy (fig. 9 *A*). The atrophic muscle showed only slight changes in the two week period of observation after the administration of the vitamin $B_6$.

18. Wolbach, S. B.: Personal communication to the authors.

*Paw.*—On the paw, after seventy-two days on diet SS, the epidermis was thickened, the cells appeared larger, with much larger nuclei and prominent nucleoli. The stratum granulosum was present but not conspicuous; the stratum lucidum was prominent. Hyperkeratosis and acanthosis were striking features. The basalis, which was well developed, contained numerous mitotic figures. The hair follicles and

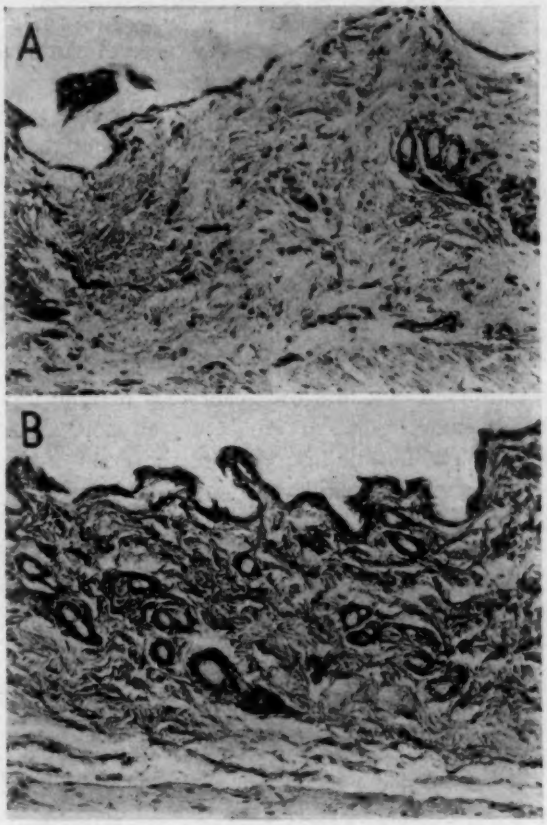


Fig. 9.—*A*, skin from a deficient animal forty-eight hours after the administration of 100 micrograms of vitamin $B_6$. Note the area containing few hair follicles. There is some attempt at hair follicle regeneration at the periphery (× 280). *B*, skin from a deficient animal ninety-six hours after the administration of 100 micrograms of vitamin $B_6$. Note the focal area of follicle regeneration (× 280).

sebaceous glands were atrophic. There were areas which were ulcerated and contained numerous polymorphonuclear leukocytes, and necrotic areas were not infrequently encountered. The blood vessels in the corium were markedly distended, and edema was a prominent feature.

Forty-eight hours after the administration of 100 micrograms of vitamin $B_6$, the stratum granulosum again became prominent. The edema disappeared, and histiocytes replaced many of the polymorphonuclear leukocytes in the picture. No effect was noted in extensively infected or necrotic areas. Healing of the paw was slower than other portions of the body. This was probably due to the constant trauma, as well as to the marked inflammation which was usually present in these regions.

*Snout.*—In the normal snout the epithelial covering is usually one to four layers deep and consists for the most part of flattened cells with a prominent stratum granulosum and a stratum corneum which is also prominent but not as extensive as those seen on the ears. After seventy-two days on diet SS, the epithelial cells became larger than normal, and the prickle cell layer was prominent. The stratum granulosum was distinct, and the stratum corneum was thickened and denser than normal. Persistence of nuclei in the stratum corneum was seen in some areas. The papillae were edematous and contained numerous round cells and congested capillaries. Mitotic figures were numerous, and ulceration was frequent.

After the oral administration of 100 micrograms of vitamin $B_6$, the epithelial layer thinned out, and the acanthosis became less prominent. Collections of polymorphonuclear leukocytes in both dermis and epidermis remained. The mitotic figures were slower in disappearing than in the ear. At times the histiocytes and round cells about the hair follicles increased in number. Secondary inflammatory lesions did not appear to be appreciably affected by the administration of the vitamin.

The ulcers which occurred at the base of the under surface of the tongue showed only a slight tendency toward healing in a two week observation period after the administration of vitamin $B_6$.

*Liver.*—Histologic sections of the livers of the deficient animals as a rule showed considerably less fat than those of the livers of animals treated with vitamin $B_6$. On the other hand, if the deficient rats received vitamin $B_6$, the livers, particularly the left lobes, often showed considerable amounts of fat. This was striking with diet SS, which did not contain choline, and occurred only rarely with diet SD, which was supplemented with choline. In only 2 rats on diet SS did we find the degeneration and cirrhosis described by György and Goldblatt [19]; and none of the rats on diet SD, which contained an adequate choline supplement, showed these conditions. This is interesting in view of the fact that György and Goldblatt [20] suggested that the vitamin $B_6$ in their diet influenced the cystine-methionine ratio which Griffith and Wade [21]

19. György, P., and Goldblatt, H.: J. Exper. Med. **70**:185, 1939.
20. György, P., and Goldblatt, H.: J. Exper. Med. **72**:1, 1940.
21. Griffith, W. H., and Wade, M. J.: J. Biol. Chem. **132**:567, 1940; Proc. Soc. Exper. Biol. & Med. **41**:188, 1939.

considered important in determining the need for choline and the fact that Earle and Victor [22] reported cirrhosis of the liver caused by excess cystine.

*Adrenals.*—When extreme symptoms became apparent, the glomerular cells contained fine vacuoles with eosinophilic granules, which apparently occurred at the intersection of the cytoplasmatic fibrils. The outer fascicular layer was wider, and the cells in this region were large. The cytoplasmic material was increased in amount and contained an occasional larger eosinophilic or basophilic granule. In the deeper layers of the fascicular and reticular zones, vacuoles of varying size were present. The reticular zone was narrow and consisted of small, dense cells. The cells showed no reticular arrangement but appeared as a compact layer. Some of the cells contained brown pigment granules. The cells of the medulla were closely packed.

Sixteen hours after the administration of 100 micrograms of vitamin $B_6$, the cells of the glomerular layer were somewhat enlarged, and the cells were finely vacuolated, giving a more distinct foam cell appearance. The general contour of this zone, however, remained fairly constant. The cytoplasm of the fascicular zone increased in amount and appeared clearer. The nucleus, which had remained constant in size, was now only one-fourth to one-fifth the diameter of the entire cell. Isolated large eosinophilic and basophilic granules, as well as diffuse fine eosinophilic granules, were present. The reticular zone appeared narrow and compact, and the cells had a homogenous cytoplasm. There was considerable congestion of the inner part of the fascicular zone and of the reticular zone. Some of the aforementioned changes are suggestive of the alarm and adaptation reaction described by Selye.[23] This progressed for from seventy-two to one hundred forty-four hours after the administration of vitamin $B_6$. The glomerular layer became prominent. The cytoplasm in the fascicular zone became clearer and less granular. The cells with brown pigment were more numerous and conspicuous. Some of the pigmented cells were elongated, resembling macrophages.

The medullary cells were larger and less dense. If no further vitamin $B_6$ was administered, the adrenals in about one to two weeks returned to a state like that found in deficient animals.

The appearance of the glomerular layer both before and after the administration of vitamin $B_6$ may correspond with the glomerular changes described by Butcher and Richards in rats with retarded hair growth.[24]

22. Earle, D. P., and Victor, J.: J. Exper. Med. **73**:161, 1941.

23. Selye, H.: Endocrinology **21**:169, 1937.

24. Butcher, E. O., and Richards, R. A.: Endocrinology **25**:787, 1939.

Adrenal hemorrhages, encountered in rats on diets deficient in pantothenic acid, were found in rats on diets SS and SD.[25] Extensive adrenal hemorrhages were found at autopsy in 12 of 100 animals on diet SD. The frequency of adrenal hemorrhages reached a peak during the fourth week, at a time when the lesions from vitamin $B_6$ deficiency were insignificant. No adrenal hemorrhages were found during the first two weeks on this diet. The hemorrhage usually started in the inner third of the cortex and had a tendency to involve first the entire cortex before destroying the medulla. In some instances, enormous vacuolated cells were found adjacent to the hemorrhagic areas. Not infrequently animals which were put to death after the fourth week without any clinical symptoms referable to the adrenals showed small areas of organizing hemorrhage, and in later periods, fibrosis and calcification. Adrenal hemorrhage did not occur in rats maintained on diet 235, which contained an adequate supply of pantothenic acid.

*Bones.*—The cartilage at the epiphyses of the bones showed increased calcification and small cells. The picture was reminiscent of that described by Silberberg and Silberberg[26] as occurring in malnourished rats and may have been a nonspecific reaction due to malnutrition rather than to vitamin $B_6$ deficiency proper. No return to the normal state after the administration of vitamin $B_6$ was noted during a two week period of observation.

*Testes.*—The deficient animals showed abnormal spermatogenesis or none at all. The spermatocytes and spermatids were enormous in size, often multinucleated. Mature spermatozoa were rarely found. The seminal vesicles, prostate and Cowper's gland were very small. All of these findings were in agreement with those of Emerson and Evans.[27] After administration of a single dose of vitamin $B_6$, no significant change was noted in the testes during the two week period of observation. However, rats maintained on a similar diet with the addition of vitamin $B_6$ showed less pronounced pathologic changes even in the absence of pantothenic acid.

Panmyelophthisis, described by György and co-workers,[28] was observed in only 1 of our rats.

## COMMENT

It is extremely difficult to delineate those changes which are due to the vitamin $B_6$ deficiency per se from the nonspecific secondary changes

25. Daft, I. S., and Sebrell, W. H.: Pub. Health Rep. **54**:2247, 1939. Nelson, A. A.: ibid. **54**:2250, 1939. Unna.[16]

26. Silberberg, M., and Silberberg, R. S.: Arch. Path. **30**:674, 1940.

27. Emerson, G., and Evans, H. M.: Am. J. Physiol. **129**:P352, 1940. Emerson, G.: Personal communication to the authors.

28. György, P.; Goldblatt, H.; Miller, F. R., and Fulton, R. P.: J. Exper. Med. **66**:579, 1937.

due to malnutrition and infection. In addition, with multiple vitamin deficiencies, cure of one manifestation may be accompanied by aggravation of the remaining deficiencies. Wolbach [29] emphasized this point by his statement, "The pathologic or morphologic characterization of a vitamin deficiency involves a dual basis: (*a*) the changes in consequence of the deficiency and (*b*) the changes accompanying recovery from the deficiency ending in restoration to normal structure." In our investigations particular attention has been paid to (*a*) the regular occurrence of the specific symptoms on the specifically deficient diet and the absence of these symptoms in animals receiving an adequate dietary supply of the vitamin in question and (*b*) the response of the deficiency symptoms to administration of small doses of the vitamin. Complete restitution of the normal appearance may not occur in all cases after the administration of the missing vitamin, owing possibly to infection or other secondary effects resulting in permanent retardation of the endocrine or other visceral functions. In cases of irreparable morphologic damage, such as hemorrhage into the adrenal, the return to normal obviously cannot occur. In instances in which the deficiency state results in a secondary metabolic disorder, the addition of an intermediate or final metabolite may result in improvement of the deficiency symptoms. A possibility such as this must be accepted, since it has been claimed that "rat acrodynia" can be influenced by essential fatty acids without vitamin $B_6$ [30] and, according to Birch,[31] it appears that vitamin $B_6$ is connected with the utilization of the unsaturated fatty acids.

Histologic studies by others on the lesions due to vitamin $B_6$ deficiency have appeared, but all of the aforementioned factors were not considered in the evaluation of the specificity of the lesions.[32]

The cutaneous findings on the ears, paws and snout are found in our rats on vitamin $B_6$–deficient diets; they occur despite the better nutritional state after the addition of pantothenic acid or vitamin $B_6$–free liver extracts; they do not occur with diets adequate in vitamin $B_6$, and the rats return to an essentially normal appearance after the administration of vitamin $B_6$. In view of the foregoing evidence these lesions may be considered specific for vitamin $B_6$ deficiency, the ear lesion being most characteristic. Sullivan and Nichols [32b] have implied that the changes in the ears are not specific for vitamin $B_6$ deficiency. This disagreement

29. Wolbach, S. B.: J. A. M. A. **108**:7, 1937.

30. Quackenbush, F. W.; Platz, B. R., and Steenbock, H.: J. Nutrition **17**: 115, 1939. Schneider, H.; Steenbock, H., and Platz, B. R.: J. Biol. Chem. **130**: 539, 1940.

31. Birch, T. W.: J. Biol. Chem. **124**:775, 1938.

32. (*a*) György, P.; Sullivan, M., and Karsner, H. T.: Proc. Soc. Exper. Biol. & Med. **37**:313, 1937. (*b*) Sullivan, M., and Nichols, J.: J. Invest. Dermat. **3**:317, 1940.

may have been due to a small amount of vitamin $B_6$ in the preparation of "filtrate factor" used in their diet.

The changes of the skin of the trunk, the adrenals and other visceral organs are not considered to be due to the vitamin $B_6$ deficiency. A relation between the testicular changes and vitamin $B_6$ cannot be ruled out.

The addition or the absence of choline does not influence the lesions ascribed to vitamin $B_6$ deficiency. The fact that a fatty condition of the liver was not found on morphologic examination of rats on the diets which were free of choline, except after the administration of vitamin $B_6$, points to the acknowledged function of choline as a lipotrophic factor with vitamin $B_6$ playing an opposite role. This would agree with György and Goldblatt's contention in their paper [20] on the development of cortical necrosis of the kidneys that vitamin $B_6$ has an aggravating influence on the specific effect of choline deficiency.

The relation between alterations of the adrenals and vitamin deficiencies is a complex one. Some of the changes, as have been mentioned, are comparable to the alarm and adaptation reaction described by Selye.[33] The correlation of the posthypophysectomy adrenal hemorrhage and atrophy reported by Selye [33] and the adrenal atrophy reported by Crooke and Gilmour [34] with the adrenal hemorrhage occurring in pantothenic acid deficiency must also be considered. Morgan and Simms [35] have attempted to correlate adrenal atrophy and senescence caused by vitamin deficiency. This possible relation is further emphasized by the exaggeration of the state due to pantothenic acid deficiency after the administration of thyroid and the slight healing of vitamin $B_6$ deficiency after the administration of anterior pituitary extract.[36]

The histology of the residual skin changes, such as the loss of the long cover coat and alopecia, the adrenal hemorrhages and the discoloration of the fur and whiskers, will be the subject of another communication dealing with pantothenic acid deficiency.

## SUMMARY

The hyperkeratosis and acanthosis of the ears, paws and snout, together with the edema of the corium, are considered characteristic of vitamin $B_6$ deficiency in rats.

33. Selye, H.: J. A. M. A. **115**:2246, 1940.
34. Crooke, A. C., and Gilmour, J. R.: J. Path. & Bact. **47**:525, 1938.
35. Morgan, A. F., and Simms, H. D.: Science **89**:565, 1939; J. Nutrition **19**:233, 1940.
36. Unpublished data.

# Case Reports

## OBTURATOR THROMBOSIS OF THE RIGHT PULMONARY ARTERY AND NECROSIS OF THE ENTIRE LUNG WITH HILAR BRONCHOGENIC CARCINOMA

E. Gordon Behrents, M.D., Chicago

The complications arising from primary carcinoma of the lung either in the region of the initial tumor or in that of metastases are many. Obturator thrombosis of the main branch of the pulmonary artery with colliquative necrosis of the entire lung secondary to primary hilar carcinoma is rare. Many of the published reports of thrombosis of the pulmonary artery include descriptions of occluding thrombi of the main stem and small intrapulmonic branches, but Kampmeier's [1] review in 1934 contained only those of the main branches of the pulmonary artery. Kampmeier collected 23 reports and added another. Since then, 19 additional records of thrombosis of the main branches of the pulmonary artery have been published, of which 8 concerned the obturative type.[2] Embolism from some focus has been considered an important cause.[3] Sclerosis and aneurysms of the pulmonary artery as a basis for autochthonous thrombosis also have etiologic significance.[4] Tumors have been considered responsible in only a few reports. Brenner found only 1 account of leiomyosarcoma of the root of the pulmonary artery with spread into the main branches. Fried's [5] report on primary carcinoma of the lung included several cases in which the tumor tissues had invaded the wall of a large intrapulmonic branch of the pulmonary artery and caused thrombosis. In none was the thrombosis complete and accompanied with necrosis of the lung. White and Brenner [6] observed invasion of the wall of the pulmonary artery by carcinoma tissues. Crawford and Mohler included in a report of 30 cases of pulmonary thrombosis

From the Cook County Hospital.

1. Kampmeier, R. H.: J. Thoracic Surg. **3**:513, 1934.

2. Cabot Case 26111, New England J. Med. **222**:457, 1940. Crawford, B. L., and Mohler, H. K.: Pennsylvania M. J. **40**:1020, 1937. Pou, J. F., and Charr, R.: Am. Rev. Tuberc. **37**:394, 1938. Schneider, H. O., and Van Ordstrand, H. S.: Cleveland Clin. Quart. **7**:284, 1940. Wade, J. L.: West Virginia M. J. **36**:69, 1940.

3. Brenner, O.: Arch. Int. Med. **56**:1189, 1935. Desclin, L.: Frankfurt. Ztschr. f. Path. **40**:161, 1930. Fowler, W. M.: Ann. Int. Med. **7**:1101, 1934. Means, J. H., and Mallory, T. B.: ibid. **5**:417, 1931. Moller, P.: Beitr. z. path. Anat. u. z. allg. Path. **71**:27, 1923.

4. Karsner, H. T.: Arch. Int. Med. **51**:367, 1933. Monckeberg, J. G.: Deutsche med. Wchnschr. **33**:1234, 1907. Posselt, A.: Ergebn. d. allg. Path. u. path. Anat. **13**:298, 1909.

5. Fried, B. M.: Medicine **10**:373, 1932.

6. White, P. D., and Brenner, O.: New England J. Med. **209**:1261, 1933.

and embolism a description of a carcinoma of the lung which compressed the pulmonary artery and caused thrombosis.

In all obturator thrombi of the pulmonary artery or its main branches, portions were organized and a few were partially calcified. Where the root or both primary branches were occluded, complete obstruction had been caused by a recent thrombus. Among the 24 cases of thrombosis in Kampmeier's report were 11 instances of bilateral thrombosis—10 of the right branch only and 3 of the left.

The changes in the lung tissues with thrombosis of the pulmonary artery were not consistent. Hyperemia was the most common condition. In some lungs there were small infarcts; in others, regions of atelectasis. Collateral circulation established through bronchial and tracheal arteries, as described by Means and Mallory, prevented gross infarction. The pulmonary condition in the cases described by Mallory, Fowler, Hart[7] and others was similar, but without enlargement of the bronchial arteries. In the case described by Karsner, collateral circulation was provided through pleural adhesions. Schneider and Van Ordstrand described extensive ischemic necrosis of the lung with obturator thrombosis of the main pulmonary artery.

The clinical symptoms of thrombosis of the pulmonary artery are usually associated with a basic disease of the heart or some other thoracic condition. In the reported cases, the onset was usually gradual, and the symptoms were those of cardiac decompensation, most frequently dyspnea, cough and cyanosis. Substernal or epigastric pain or a sensation of pressure, hemoptysis and restlessness were common complaints. The lungs gave the signs of passive hyperemia, occasionally impaired breath sounds or percussion notes, and a friction rub over the regions with infarcts. Blood-stained pleural fluids also were found. These were the salient signs and symptoms suggested by Kampmeier for a clinical diagnosis.

## REPORT OF A CASE

A Negro aged 75 years entered the dispensary of St. Luke's Hospital Dec. 27, 1939, because of weakness, anorexia, dyspnea and loss of weight during six months. He had a hyperresonant chest but no signs of cardiac disease. The blood pressure was 200 systolic and 100 diastolic. The weight was 145 pounds (65.5 Kg.). Analyses of the blood and urine showed nothing unusual. The upper portion of the right lung in a roentgen film was incompletely aerated. When the patient was examined again, Jan. 10, 1940, there was dulness posteriorly over the lower and middle lobes of the right lung. He was admitted to the hospital June 27, because of dyspnea, cough, loss of weight, weakness and anorexia. He did not seem acutely ill; his pulse rate was 110 and his respiratory rate was 32 per minute. A small hard nodule was present in the subcutaneous tissues above and lateral to the right nipple. A roentgen film demonstrated opacity of the right side of the chest and deviation of the mediastinum to the left. The leukocytes of the blood numbered 11,640 per cubic millimeter. The urine had 40 mg. of albumin per hundred cubic centimeters. On the second day, the temperature rose to 105.2 F., the respirations ranged to 36 per minute and the patient coughed frequently and appeared cyanotic. July 29, by thoracentesis, 1,000 cc. of a black-

7. Hart, C.: Deutsches Arch. f. klin. Med. **134**:449, 1905.

brown fluid was removed from the right side of the chest. By a second thoracentesis, July 30, 400 cc. of a similar fluid was removed. The patient's condition remained the same; he was dyspneic, and died six days after admission to the hospital.

*Autopsy* (by Edwin F. Hirsch).—The body was 160 cm. long and weighed 115 pounds (52 Kg.). In the right anterior axillary line at the fifth interspace was a firm subcutaneous nodule of gray tissue, 1.5 cm. in diameter, and nearby was another, 6 mm. in diameter. The entire right lung (figure) except the firm hilar structures was liquefied, and the pleural space contained 2,080 Gm. of black fluid and autolyzed lung tissue with only a slight odor. The hilar residue of the right lung tissues was 8.5 cm. long, 5 cm. thick and 6 cm. wide. Only the tougher portions of the main bronchi, covered with tags of necrotic black lung

Photograph of the obturator thrombus of the right branch of the pulmonary artery. The right main bronchus is above and to the left; the opened pulmonary vein is to the right.

tissues, remained. Some of these bronchi opened directly into the pleural space. The right branch of the pulmonary artery extended into the necrotic hilar tissues. The lumen was dilated and occluded by a blood clot 3 cm. in diameter and about 3.5 cm. long. The proximal end of the clot was flat, and it reached into the main branches of the pulmonary artery, filling their lumens. The lymph nodes below the bifurcation of the trachea, black with carbon, were 2.5 by 3.5 cm. in maximum surface dimensions. The pulmonary veins, opened from behind, contained postmortem blood clots and fluid blood. The left lung weighed 510 Gm. The posterior portions were edematous; the pleura was smooth and black with carbon. Surfaces made by cutting the lung were crepitant, gray or hyperemic, and without nodules of tumor. The lining of the bronchioles in this lung was hyperemic; the lining of the branches of the pulmonary vein and the pulmonary artery was smooth.

The trachea had no changes. The routine postmortem examination of the head, neck and trunk demonstrated no other tumor tissues in the viscera. Each kidney had a small recent infarct.

*Histologic Examination.*—Preparations of the large divisions of the right main bronchus were examined. In one, the tunica propria of the mucosa was markedly thickened by fibroplastic tissues, and the cells of the muscularis mucosae were hypertrophied. These thickened stroma tissues lying between the lumen and the cartilage plates were invaded by masses of carcinoma, some in bizarre tubules, others in mosaics of pavement cells. The neoplasm extended into the outer portions of the wall and also had penetrated the lymph channels there. These tissue relations agree with the structure of small growths of carcinomas arising in the main divisions of a bronchus.[8] Sections of the obturator thrombus of the right branch of the pulmonary artery contained organizing granulation tissues and glandular carcinoma. The two subcutaneous nodules near the right axilla were lymph nodes, also with metastatic glandular carcinoma. Histologic preparations of the left lung and other viscera showed no carcinoma.

This is an unusual complication of primary hilar carcinoma of the lung. Invasion of the wall of pulmonary vessels by carcinoma occurs frequently and usually has no clinical importance except that involvement of the veins is associated with widespread metastases. In this patient the tumor invaded the main right branch of the pulmonary artery, causing thrombosis, and apparently caused occlusion of the bronchial arteries so that no collateral circulation was established. Ischemic colliquative necrosis of the entire lung resulted. Thus, thrombosis of the pulmonary artery secondary to the hilar carcinoma caused necrosis of the lung and destroyed most of the tumor as well.

Although a peripheral tumor of the right lung could invade hilar structures and cause the lesions described, the position and distribution of the carcinoma tissues in the tunica propria of the hilar bronchiole support the view that this is probably the primary focus.

SUMMARY

A hilar bronchogenic neoplasm diagnosed as carcinoma caused obturative thrombosis of the right main branch of the pulmonary artery with colliquative necrosis of the entire right lung. There seems to be no other record of such a complication with bronchogenic carcinoma.

8. Thomas, H. B.; Hirsch, E. F., and Blaine, E. S.: J. A. M. A. **80**:89, 1928.

# Laboratory Methods and Technical Notes

## PHOTOMICROGRAPHY WITH 35 MM. KODACHROME

JOSEPH C. EHRLICH, M.D., NEW YORK

The results obtained during the past year in over a thousand exposures have offered convincing evidence that photomicrography with 35 mm. Kodachrome film can yield results which are almost completely satisfactory with regard to rendition of color and detail at various magnifications. These transparencies when suitably projected, even to large diameters, yield excellent, brilliant images. Photomicrography on Kodachrome film has been successfully practiced for several years with cut film sizes, but it can now be stated that fine histologic details, such as intracytoplasmatic granules of neutrophilic leukocytes and intranuclear structural details, can be rendered sharply and in satisfactory color even when condensed within the narrow confines of the 35 mm. transparency.

The apparatus assembly is somewhat more complicated than that necessary for ordinary black and white photomicrography, but the actual operation of a completely assembled and standardized outfit is much simpler than that for black and white photography and can be made to yield uniformly satisfactory results. The time and the expense involved in development, drying, printing and mounting are unnecessary. The color rendition compares favorably with general Kodachrome photography in yielding images which closely approximate in natural color the specimen employed.

The approach to photomicrography with 35 mm. Kodachrome film is somewhat different from that to black and white photomicrography and requires attention to certain basic principles in order to obtain uniformly satisfactory results. The quality of the color rendition depends on three main factors. These are (1) the original preparation to be photographed, (2) the apparatus assembly and (3) the exposure.

*Original Preparation to be Photographed.*—Excellent rendition of color is naturally conditioned by the microscopic slide preparation used, and it is fruitless to attempt to produce brilliantly colored transparencies from preparations which, to begin with, are poorly sectioned and poorly stained.

*Apparatus Assembly.*—Details of the apparatus assembly which require careful consideration from the standpoint of color rendition are: (*a*) the source of incandescent light, (*b*) the filters and (*c*) the color-corrected optical system.

(*a*) Source of Incandescent Light: Various sources of incandescent light suitable for Kodachrome photomicrography are commercially available. Homemade assemblies utilizing photoflood lamps have also been

From the laboratories of Lebanon Hospital and Mount Sinai Hospital.

tried. In general, a strong light source with a concentrated filament operating at a low or a standard voltage is advised. The lamp should produce light of a color temperature in the neighborhood of 3,450 kelvins and should be fitted with a ventilated housing, a condensing lens and an iris diaphragm.

(*b*) Filters: Extensive experience with Kodachrome photomicrography has demonstrated conclusively that best results can be obtained only with the use of correct filters. This applies particularly to the rendition of preparations stained with eosin. This is true even when the color temperature of the light source is ideal. The difficulty is due to certain peculiarities of the dye itself, to the fact that the film is corrected for use with reflected and not transmitted light and to color band absorptions by the glass in the optical elements themselves.

Color temperature correction filters (Eastman Kodak Company series CC 3, 4, 5, 6, 13, 14, 15) fail to correct for this difficulty. The magenta series (Eastman Kodak Company CC 33, 34, 35) is better but also inadequate. Other dyes with various absorption bands which we have tried, including congo red, sudan IV, cobalt sulfate, phenolphthalein, phenolsulfonphthalein, oxyhemoglobin, methyl orange, orange G, eosin, potassium dichromate and others, are likewise unsatisfactory.

Neodymium or didymium (neodymium + praseodymium) filters offer satisfactory correction of the spectrum from an incandescent light source, particularly for eosin and other pink stains. These filters produce a narrow region of absorption of yellow from an incandescent light source (0.589 micron sodium spectral line, 0.578 micron mercury spectral line), thus accentuating pink colors. Neodymium is available as neodymium carbonate (Maywood Chemical Company, Maywood, N. J.) for use in solution in absorption cells. Didymium is available as didymium glass (Corning no. 512 and no. 592).

(*c*) Color-Corrected Optical System: For best results, color-corrected optical elements should be used throughout. An aspheric lamp condenser, an aplanatic or an achromatic substage condenser, apochromatic objectives and compensating eyepieces are recommended. Optical elements which are less adequately corrected for color produce results which are relatively lacking in detail but which may be within satisfactory limits. All bluish or greenish glass, such as that in microslides, filters, diffusion glasses and absorption cells, must be strictly avoided.

*Exposure.*—Exposure must be *exact*. The wide latitude of exposure which can give satisfactory results in black and white photography does not apply to Kodachrome film, and there is no opportunity to correct for overexposure or underexposure of the negative by the use of intensifiers or reducers, or by corrective printing. With Kodachrome film, slight underexposure produces an over-all opacity and intensification of blues. Slight overexposure washes out blues and red and produces thin, indistinct yellowish brown transparencies. It is therefore of first importance to have some means of determining accurately and within very narrow limits the correct exposure. The method adopted here to insure accurate exposure is not claimed to be the only method by which this may be determined. No experience on our part has been acquired in the establishment of accurate exposure by photoelectric determination.

The method used to establish accurate exposure is to predetermine the effective arrangement of, and fix, every unit in the apparatus assembly which may vary the quantity of light which ultimately reaches the film. These units which require standardization are: (1) the light source, (2) the light source diaphragm, (3) the filter, (4) the distance of the light source from the substage mirror, (5) the level of the substage condenser, (6) the aperture of the substage iris, (7) the objective, (8) the eyepiece and (9) the distance between the eyepiece and the film. Standardization of these factors is accomplished in the following way:

1. Light Source: Because of voltage variations in the hospital circuit, a 6 volt 5 ampere lamp is used with a storage battery current source. The storage battery is kept carefully filled and on a continuous trickle charger, so that a constant voltage and a maximum battery output are maintained at all times.

2. Light Source Iris: The light source is fitted with a calibrated iris diaphragm. The aperture of this iris is fixed for each objective and is always used at the same calibration.

3. Filter: A single filter is used (see section on filters) and is always in place for each exposure.

4. Distance of the Lamp from the Substage Mirror: The microscope and the lamp are fixed in permanent distance relationship, and the distance between them is never changed.

5. Substage Condenser Level: A scale is fixed to the substage apparatus, and the level of the substage is always placed at a constant point.

6. Substage Iris Diaphragm Aperture: The optimum aperture for each objective is predetermined and fixed for each objective. This aperture is always used at the same calibration for each objective.

7. Objective: The same objective is always used for each magnification.

8. Eyepiece: The same eyepiece is always used for each magnification.

9. The Distance between the Eyepiece and the Film: This distance is fixed for all magnifications and never changed.

Under the conditions just outlined the only variable element introduced is the preparation itself. A uniform grade of slides, of mounting mediums and of cover glasses reduces this variation to a minimum, and the only other factor which might have to be taken into account is the variation in the intensity of staining and the density of the preparation. This factor introduces not more than a 10 per cent variation in exposure, which can be regulated empirically after some experience.

After the apparatus assembly has been completed and its component parts set in fixed positions according to the foregoing outline an object is selected for test exposures. For this purpose, a brightly stained, well differentiated hematoxylin-eosin preparation is suitable. With the 16 mm. objective, a series of test exposures is made empirically. Suitable tests would be $\frac{1}{100}$, $\frac{1}{50}$, $\frac{1}{25}$ and $\frac{1}{10}$ second. With the 8 mm. objective, $\frac{1}{50}$, $\frac{1}{25}$, $\frac{1}{10}$ and $\frac{1}{5}$ second may be tried. With the 4 mm. objective, $\frac{1}{10}$, $\frac{1}{5}$, $\frac{1}{2}$ and 1 second, and with the 90 × oil immersion, $\frac{1}{5}$, $\frac{1}{2}$, 1 second, 2 seconds and 4 seconds may be tried. If a series of test exposures through a suggested range does not produce correctly exposed transparencies, a new series of tests is carried out at another range. Careful notes must be kept of each test exposure. The transparencies

as returned are numbered in serial order and can be checked against their respective notes. When the optimum exposure is determined for each objective, all subsequent exposures with that objective are made at the same interval.

Certain principles must be given attention: (*a*) The microscope body tube, the eyepiece holder and the camera must be completely free of interior reflections.

(*b*) Where a vertical apparatus is used by means of a substage reflecting mirror, a substage prism may be employed instead of a substage mirror in order to avoid the splitting of the beam from the front and rear surfaces of an ordinary mirror and to provide against light loss and light beam distortion by ordinary mirror glass.

(*c*) Because of the intensity of the light source used, the apparatus should be equipped with a shutter that will permit short exposures. It has been found that a focal plane shutter is preferable to a leaf shutter because the latter sometimes produces "shutter-kick" images at short exposures. The shutter should furthermore be in excellent mechanical condition, so that it always produces an exposure of exactly the same interval at each setting of the shutter. However, if the only shutter available cannot give short exposures, the exposure may be prolonged without changing the color rendition by the use of neutral density filters (these must be checked at the Eastman Kodak Company Laboratories in Rochester, N. Y., for correct color transmission). The intensity of the light cannot be cut down by the use of a resistance factor, because the color temperature of the light will be altered. It is also inadvisable to use a ground glass to cut down the intensity of the light, because the color correction of the beam and the color temperature may be disturbed.

(*d*) All focusing must be done critically. Since the distance of the film from the eyepiece must be small (about 12 to 20 cm. with a 10 × eyepiece), the fields are reduced to a very small size, and a magnifying lens should be used in focusing the image on the ground glass. The slightest inaccuracy in focusing will become painfully evident when the transparency is projected. As in all miniature photography, the slightest defects become conspicuous during projection. This necessitates attention to such details as the removal of dust from the lens and the film carrier. The film carriage must not scratch the film.

The particular form of the film holder and of the focusing mechanism may be selected according to individual preference. I have been using the Miflex photomicrographic attachment, which provides a direct ground glass image before exposure without cumbersome interchange of parts. A Contax camera and a Contax adapter for Miflex are used for carrying the film. The apparatus standardized according to the principles mentioned in this paper will, after short experience, yield consistently successful results.

# General Reviews

## MACROMOLECULAR SUBSTANCES AS PATHOGENIC AGENTS

W. C. HUEPER, M.D.
NEW YORK

Macromolecular substances of varying physicochemical properties and composition are not only of profound biologic significance as more or less stable building materials of the different intracellular and extracellular structures and secretions of the animal and the vegetable organisms but are of importance in the normal and pathologic vital dynamics of living matter. The following four chemical groups of macromolecular compounds may be distinguished: (1) proteins, such as albumins, globulins, fibrinogens, hemoglobins, gelatin, silk fibroin and edestin; (2) carbohydrates, such as glycogens, starches, celluloses, pectins, mucopolysaccharides, gums, mucilages, chitins and lichenins; (3) lipins, such as polydiaminophosphatides; (4) combinations of these basic macromolecules, such as lipoproteins, glycoproteins (nucleoproteins) and glycolipids. Modern chemistry has added in recent years to this list of natural or biogenic macromolecules an appreciable and ever increasing number and variety of synthetic macromolecular polymers, such as are used in artificial rubbers, textile fibers, plastics and resins. Thus there exist macromolecular substances composed of heterogenic members, such as proteins, and others consisting mainly or exclusively of homogenic members, such as the polysaccharides and the majority of the synthetic highly polymerized compounds. Depending on the number and the types of the component chemical groups, as well as on the arrangement and the bindings of these groups within the molecule, macromolecular compounds vary greatly in molecular size and configuration.

The accompanying table presents a number of substances with molecular weights of above 1,000.

These figures, which represent approximations of the molecular weights of the various compounds, are based in general on measurements made by ultrafiltration or ultracentrifugation (Svedberg) (Stanley; Gowen; Stern and Duran-Reynals; Bechhold; Liesegang; Staub and co-workers; Hitchcock, and others) or are calculated by applying Staudinger's formula concerning the relation between the degree of viscosity of long-chained compounds and their molecular weight. It is

From the Warner Institute for Therapeutic Research.

remarkable that all the autogenous pathogenic macromolecules, the viruses, are of giant size; i. e., they possess molecular weights above 1,000,000.

The extent to which the degree of polymerization influences the molecular weight is apparent from the following data: A certain type of methyl cellulose composed of approximately 400 units of dextrose has a molecular weight of 72,000 (Sowden and Lasettre); cellulose, on the other hand, is said to consist of 1,000 to 2,000 of such molecules (Staudinger; Freudenberg); in the various types of glycogen the degree of polymerization varies from four hundred to five thousand times, and in those of starch, from one hundred eighty-five to nine thousand times; a

*Molecular Weights of Macromolecular Compounds*

| Substance | Molecular Weight | Substance | Molecular Weight |
|---|---|---|---|
| **Proteins:** | | **Carbohydrates:** | |
| Lysozyme | 18,000 | Inulin | 2,430 |
| Egg albumin | 34,000 | Dextrin | 4,400 |
| Hemoglobin | 68,000 | Soluble starch | 10,000 |
| Serum albumin | 72,000 | Emulsin | 37,700 |
| Diphtheria toxin | 72,000 | Invertin | 44,900 |
| Pseudoglobulin | 80,000 | Starch | 30,000 to 153,000 |
| Casein | 98,000 | | |
| Euglobulin | 140,000 | Cellulose | 172,000 to 344,000 |
| Gelatin | 150,000 | | |
| Serum globulin | 175,000 | Glycogen | 283,000 to 344,000 |
| Vegetable globulins (edestin, excelsin, almadin) | 200,000 to 300,000 | Pectin | 150,000 to 300,000 |
| Antisheep hemolysin | 420,000 | | |
| Thymonucleic acid | 200,000 | | |
| Hemocyanin | 6,700,000 | | |
| Tobacco mosaic virus | 50,000,000 | **Lipins:** | |
| Various plant disease viruses | 34,000,000 to 88,000,000 | Triaminophosphatides | 1,400 |
| | | Protargon | Above 6,000 |
| Bacteriophage | 25,000 to 36,000,000 | **Synthetic Compounds:** | |
| Rabbit papilloma virus | 25,000,000 | Polyvinyl alcohol | 17,000 |
| Chicken tumor virus | 139,000,000 | Methyl cellulose | 50,000 |
| Fowl leukemia virus | 139,000,000 | Polystyrol | 840,000 |

certain sample of polyvinyl alcohol examined by Dammann, Lange, Bredig and Nord was composed of 300 vinyl alcohol molecules, having thus a molecular weight of approximately 17,000, while polystyrol, studied by Staudinger, exhibited polymerization to six thousand times. It is noteworthy that the degree of polymerization exerts a deciding influence on the chemical reactivity as well as on the solubility, dispersion, precipitability, diffusibility and viscosity of a particular type of compound. Thus, an increase of polymerization is followed by an elevation of viscosity and by a decrease of solubility and dispersibility, entailing sometimes a change from a state of macromolecular colloidal solution to a micellar one. Mention may be made also of the fact that such variations are sometimes reflected in the type of reaction given with iodine or with certain dyes. Thus amylose gives a deep blue and amylopectin a blue violet reaction with iodine. With increasing decomposition

of starch, the color turns violet red, then wine red and finally yellow red. Related polysaccharides react accordingly in similar fashion to iodine (inulin, yellow; liver glycogen, deep chestnut brown; muscle glycogen, violet). Similar conditions apparently determine the variations in staining reaction given by the different types of amyloid on exposure to methyl violet. In such responses adsorption and dispersion phenomena are involved, which in turn depend on molecular size and shape.

Many of the calculated molecular weights of these megalomolecular substances had to be revised upward when it was shown by the application of roentgen diffraction spectrums and by electronic microscopic studies that such molecules are not invariably of spheroid shape but not infrequently are straight or branched, rodlike, long-chained formations (Stanley; Freudenberg; Liesegang; Bechhold; Staudinger, and others). Thus it is apparent that the diameter of macromolecules is an index neither of their size nor of their shape. The molecule of the tobacco mosaic virus in cross section is 15 millimicrons wide and 330 millimicrons in length, while the molecule of polystyrol measures 1.5 millimicrons in width and 1.5 microns in length—i. e., it is one thousand times as long as it is wide. The molecules of bacteriophages vary from 4 to 40 millimicrons in diameter, while those of several plant disease viruses range from 15 to 27 millimicrons in diameter. The macromolecules of the chicken tumor I virus measure 70 millimicrons in cross section and those of the Shope papilloma virus 40 millimicrons. The diameters of the molecules of certain starches (emulsin, invertin, soluble starch, dextrin, inulin) vary from 2.65 to 9.76 millimicrons whereas the diameter of the acacia molecule is 11 millimicrons. It is evident that the size of the pores in vascular walls and in other filtration membranes as well as the width of capillary lumens control the movements of macromolecular substances in the living organism. On the other hand, the molecular shape, apart from the molecular size, not only exerts a similar influence but determines to some extent the physicochemical relations which may develop and the reactions which may ensue between a particular macromolecular compound and its biologic environment.

Since the skin and the mucous membranes of the respiratory and the alimentary tract are impermeable under normal conditions to macromolecular compounds, disease-producing macromolecular substances either are endogenous products of a metabolism faulty as to synthesis or as to enzymatic degradation or are substances that have entered the organism from the parenteral route in the course of therapeutic, accidental or experimental procedures or as the result of a pathologic permeability of the aforementioned surface linings. Whenever substances of this type are produced in or are introduced into the organism

and whenever the body is incapable of degrading and eliminating them rapidly, they are retained in various organs and tissues because of their macromolecularity and give rise to characteristic changes and reactions in the blood as well as in the cellular structures in which they are deposited and stored.

An analysis of the functional, physicochemical and morphologic disturbances associated with certain diseases reveals the fact that the macromolecularity of compounds of different chemical composition and physicochemical properties either plays an important role in the causation of or the mechanical production of these diseases or is responsible for a part of the pathologic manifestations considered characteristic of them. Among disorders of this type are several storage diseases, such as proteinoses (e. g., amyloidosis), glycogenoses (e. g., von Gierke's disease) and probably also several of the lipoidoses (Tay-Sachs amaurotic idiocy, Gaucher's disease, Niemann-Pick disease). Hematologic as well as morphologic reactions accompanying immunity states and allergy are in part attributable to the macromolecular nature of the antigen-antibody complex formed and operative in the production of these conditions and to the existence of hyperglobulinemia, i. e., a disturbance of the macromolecular equilibrium of the plasma, found in such conditions. In this connection, mention must be made also of the megalomolecular character of certain viruses or virus proteins causing degenerative as well as proliferative (neoplastic) tissue responses (Stanley; Chase and Landsteiner; Gowen). Observations made incidental to therapeutic and experimental parenteral introduction of macromolecular compounds (proteins, glycogen, starch, acacia, hemocyanin, cellulose sodium disulfonate, polyvinyl potassium sulfate, polyanethol sodium sulfonate, lignin sulfonate, ammonium lignin sulfonate, methyl cellulose, polyvinyl alcohol, pectin, and others) (Chargaff and Olson; Demole and Reinert; Elsner, Broser and Bürgel; Eisler; Staub, Mezey and Golandas; Hueper), causing disturbances in the balance, production, degradation and elimination of the macromolecular constituents of the cells and tissue fluids, confirm the conception in regard to the significance of macromolecularity in causal and symptomatic respects.

Inasmuch as the numerous observations made concerning the part which macromolecular compounds play in the production of these pathologic reactions have remained so far mainly disconnected facts and have not led to the establishment of a macromolecular symptom complex and of a concept of macromolecular diseases, the following review is presented in an attempt to direct attention to these symptomatologic and etiologic entities. This review, however, does not include the macromolecular virus proteins, which possess autocatalytic qualities essentially lacking in pathogenic macromolecular substances of the ordinary type.

## CHEMICAL ASPECTS

It does not seem to be necessary to give in this connection a detailed description of the chemical composition and of the physicochemical properties of the macromolecular substances giving rise to macromolecular disturbances of endogenous origin (proteins, glycogen, lipins), as this information is available in many medical textbooks. It may be mentioned, however, that the pathogenic endogenous macromolecular substances apparently differ in some physicochemical respects from their normal prototypes. Thus pathogenic glycogen is less soluble in aqueous solvents than normal hepatic glycogen and is relatively refractory to the action of diastatic ferments. The proteinic amyloid differs chemically as well as in staining reactions from other proteins. The chemical nature and the physicochemical properties of the four megalomolecular substances (acacia, pectin, polyvinyl alcohol, methyl cellulose) which have been used in recent years for the experimental production of macromolecular diseases and which have been employed therapeutically in part (acacia, pectin, polyvinyl alcohol) as osmotically active colloidal agents in the control of shock, increased cerebral pressure and nephrotic hypoproteinemia in man deserve, on the other hand, a more thorough presentation.

Acacia is a polysaccharide obtained in the form of yellowish pearls as a wound secretion of certain trees and is composed mainly of arabinose and galactose molecules in addition to various hexoses, pentoses and uronic acids. Within certain limits, the chemical character of this product varies with the source of supply, the individual lot and the conditions of handling, which sometimes favor the occurrence of hydrolysis. Watery solutions of acacia are viscous. The degree of viscosity depends on the concentration of the substance in the solution and on the temperature of the solution; it decreases with rise in temperature. When the acacia is present in higher concentrations (15 to 25 per cent), these colloidal solutions are slightly turbid, brownish yellow and opalescent. They do not react with compound solution of iodine U.S.P. (Lugol's solution). They are miscible with glycerol, propylene glycol, saturated ammonium sulfate solution, saturated sodium thiosulfate solution and methyl alcohol without evident precipitation of any of the acacia. On the other hand, the addition of sufficient amounts of ethyl alcohol to the solutions of acacia causes immediate development of a stringy white spongy precipitate.

Polyvinyl alcohol is a synthetic product representing the polymerized form of the unsaturated vinyl alcohol $(CH_2\text{-}CHOH)_n$ and is chemically a ketose occupying a position between sugar and starch. Its long-chained macromolecule lacks the acid-binding groups of the protein molecule. The molecular size and the physicochemical qualities of this polymerized substance depend on the method of production and

the degree of polymerization. Polyvinyl alcohol is a tasteless and odorless white powder. Its colloidal aqueous solution often has a yellowish-greenish tint and is opalescent. The viscosity of the solution increases with the concentration of polyvinyl alcohol, with the degree of polymerization of the polyvinyl alcohol used and with fall in temperature. Gel formation occurs at a low temperature. Polyvinyl alcohol is soluble in water, glycol and glycerol and insoluble in ethyl alcohol and methyl alcohol, by which it is precipitated as a stringy white mass. It is not miscible with xylene, ether or chloroform. It is precipitated from an aqueous solution as white floccules by the addition of various salts (sodium thiosulfate, magnesium sulfate, ammonium sulfate, sodium sulfate and so on) and by organic acids (tannic acid and others). When an aqueous solution of polyvinyl alcohol is whipped with a rod, the polyvinyl alcohol is precipitated in strings and threads and collects around the rod, similar to the fibrin from identically treated plasma. Polyvinyl alcohol serves as a protective colloid in the preparation of colloidal solutions of metals. It gives some of the reactions characteristic of carbohydrates (test of Carletti and the reaction of Fleig and Ihl). On addition of compound solution of iodine U.S.P. to a solution of polyvinyl alcohol, a blue-colored gummy precipitate develops (starch reaction). The blue color fades on heating and returns on cooling. Films of polyvinyl alcohol are impermeable to oils, fats, greases and many gases. Polyvinyl alcohol solutions form emulsions with blood plasma.

Methyl cellulose is the methyl ether of cellulose. It consists of methylated dextrose molecules polymerized in a long-chained formation. The size of the molecule varies greatly with the type of methyl cellulose, and this is reflected by the marked variation in the viscosity of the solutions of these compounds. The viscosity of seven types of methyl cellulose in 2 per cent solution ranges from an average of 15 centipoises to one of 4,000 centipoises. The average molecular weight of a medium viscous methyl cellulose, having a viscosity of 50 centipoises, is about 50,000, according to information received from the Dow Chemical Company. The methyl cellulose referred to later on is of the extra high viscous type, having a viscosity of 400 centipoises. Methyl cellulose is a white, light, fluffy fibrous material and is readily soluble in water, giving a water-clear, colorless colloidal viscous solution. Solutions of concentrations higher than 1 per cent are slightly opalescent. These solutions gel when heated to temperatures above 45 C. and become firm milky coagulums at temperatures above 65 C. This coagulation by which methyl cellulose comes to resemble blood serum is, however, reversible on cooling. The coagulation reaction can be prevented by the addition of soluble salts of iodine and of sodium thiocyanate, agents which, incidentally, hinder the precipitation and deposition of lipids out of solution and which obviate the development of experimental cholesterol atheromatosis

in rabbits. Methyl cellulose solution is miscible with moderate amounts of ethyl alcohol and propylene glycol, while methyl alcohol and glycerol added to methyl cellulose cause the development of slight opalescence and gelation. Methyl cellulose is precipitated by strong inorganic and organic acids and by concentrated solutions of various salts. The same concentration of ammonium sulfate which precipitates euglobulin out of plasma also precipitates methyl cellulose completely out of its aqueous solution. Methyl cellulose gives with compound solution of iodine a dark orange brown-red precipitate, as does glycogen. Its solution forms with gelatin solution (Heller) and with blood plasma an emulsion which can be broken by prolonged centrifugation. Films of methyl cellulose are impermeable to greases and oils of vegetable and animal origin.

Pectins are a group of macromolecular carbohydrate-like substances consisting of a long-chained compound of galacturonic acid molecules with various additional groups, such as galactose, araban, acetic acid and methyl alcohol. The purified product is a fine white or slightly yellowish or brownish white powder; it is readily soluble in water, giving a colloidal, viscous solution having an acid reaction ($p_H$ 3.8 to 4.2). This solution gels on the addition of alcohol but is not stable when subjected to prolonged heating or when brought to a neutral or an alkaline reaction. A brownish precipitate is formed under these conditions, and the solution loses greatly in viscosity. This change is caused by progressive depolymerization of the pectin molecule. Pectin solutions are highly sensitive to the addition of calcium salts; this procedure leads to the formation of a water-insoluble calcium pectinate. A pectin solution is an excellent emulsifying agent for gases and liquids. Pectin is stained a cherry red with ruthenium red.

From the foregoing data it is obvious that these four exogenous macromolecular substances, which have been parenterally introduced for experimental purposes in large amounts and over considerable periods into rats, rabbits and dogs (Hueper) and which have been used in part for therapeutic purposes in man, display in many physicochemical respects great similarities to the endogenous megalomolecular substances. The animal organism does not possess normally a metabolic mechanism for the disintegration of these in part chemically relatively inert and therefore nontoxic megalomolecular exogenous substances and therefore there is a prolonged presence of these agents in an unchanged form in the organism.

## HEMATOLOGIC MACROMOLECULAR SYNDROME

Glycogenosis, or von Gierke's disease, which is characterized by a congenital inability of the body to metabolize glycogen, is accompanied in some cases by marked increase of the glycogen and cholesterol content of the blood. Chrometzka and Brunsen as well as Unshelm

recently supplied circumstantial evidence indicating that the pathologic refractoriness of glycogen toward diastatic influences is due to the formation of an abnormal glycogen-protein complex, in which the proteinic component protects the glycogenic macromolecule against the action of the diastase. Under such conditions there occur anemia, marked acceleration of erythrocytic sedimentation, neutropenia with lymphocytosis, hypofibrinogenemia and a hemorrhagic diathesis (Biedermann and Hertz; von Gierke; Unshelm; Krakower; Beumer; Wagner; van Creveld). Staub and co-workers showed that in rabbits glycogen or starch injected intravenously causes transitory leukopenia. Repeated intravenous introduction of these polysaccharides leads to the development of myeloid leukocytosis. Under these conditions the injected glycogen remains in the plasma and does not enter the leukocytes, where it is normally found (Staub, Mezey and Golandas; Golandas; Bucher and Staub). Hyperglycogenemia is thus the cause of changes in the blood which reflect a disturbance of the macromolecular plasmatic equilibrium. The coexistence of hypercholesteremia suggests that this status may give rise to an impairment of the oxygen metabolism.

Similar hematologic reactions are associated with certain transient or persistent disturbances of the protein content of the blood plasma, such as occur during allergic reactions or immunologic responses in connection with hyperglobulinemia (Schittenhelm). In anaphylactic shock the increase in the protein content of the blood is accompanied by leukopenia, relative lymphocytosis, thrombopenia followed by hyperthrombocytosis, and eosinophilia, while in infectious and immune conditions similar changes are observed in addition to accelerated sedimentation of the erythrocytes and increase in the fibrinogen content of the plasma followed by increase of globulin and albumin. Heinlein and Heinlein and Muschallik emphasized the similarity between the hematologic effects elicited by hyperergic-allergic reactions (thrombopenia, impairment of clotting, acceleration of erythrocytic sedimentation, leukopenia, hyperproteinemia, reduction of fibrinogen) and those following the intravenous introduction of macromolecular colloidal proteins, such as hemocyanin (Eisler). It is of significance that similar blood changes occur in patients showing from various causes persistent hyperglobulinemia (Bing and Plum).

This observation is of special importance, as the development of a protein storage disease (proteinosis), namely amyloidosis, not only is characterized by the presence of similar hematologic reactions (anemia, acceleration of erythrocytic sedimentation, increase in viscosity of serum) but is accompanied by more or less marked hyperproteinemia and especially hyperglobulinemia, which may become occasionally so excessive that the globulin portion of the plasma will separate from the nonglobulin part on standing (Bing; Ecklund and Reimann;

Reimann, Koucky and Ecklund; Magnus-Levy; Morgenstern). This disease, which usually appears in man in connection with chronic suppurative conditions or disorders associated with protein wastage or with disturbances of protein metabolism (osteomyelitis, chronic tuberculosis, syphilis, lymphogranuloma, myeloma), leads to extracellular and intracellular deposition of a complex proteinic substance (amyloid), especially in and around vascular and capillary walls (Hass and Schulz). As amyloidosis has been produced experimentally by repeated and protracted parenteral introduction of certain foreign proteins into animals (horses used for the production of antiserum, rabbits, mice and others) or by feeding animals an unnatural proteinic diet (Jaffé; Grayzel; Jacobi; Warshall, Bogin and Bolker; Kuczinski; Ku and Simon, and others), amyloid has been related to excessive formation of a normal or an abnormal antigen-antibody complex (Letterer; Loeschke; Primgaard; Lucké and Markley) or has been considered to be the result of a reaction of hypersensitivity. It may be mentioned in this connection that the investigations of Ehrström have shown that a mixture of serum with chondroitin sulfuric acid displays properties similar to those of amyloid and that chondroitin sulfuric acid apparently precipitates albumin, causing thereby a relative increase of the serum globulin fraction. The hematologic reactions associated with hyperproteinemia or hyperglobulinemia and with the disturbance of the colloidal equilibrium of the plasma caused thereby are thus similar to those found in hyperglycogenemia.

It may be mentioned that similar blood changes (leukopenia or leukocytosis, anemia, thrombopenia, acceleration of erythrocytic sedimentation, hemorrhagic diathesis) are observed in various lipoidoses (Gaucher's disease, Niemann-Pick disease) in which the blood plasma contains either an excessive amount of colloidally dispersed lipins or in which possibly macromolecular lipin complexes (lipoid polymers, lipoid-protein complexes) may be present (Eppinger; Müller; Epstein; Schleussner and Schnee; Kveim, and others). Chargaff pointed out that an anticoagulant attached to the sphingomyelin fraction can be extracted from the spleen in Niemann-Pick disease.

Recent experiments with a number of exogenous macromolecular substances provide additional extensive evidence in support of the macromolecular causation of these hematologic reactions. In a series of studies on macromolecular anticoagulants Chargaff and Olson noted that the intravenous introduction of the sodium salt of cellulose disulfonic acid and of the potassium salt of polyvinyl sulfuric acid is followed by marked impairment of the clotting qualities of the blood. Similar results were obtained by Demole and Reinert with the intravenous injection of polyanethol sodium sulfonate, lignin sulfonate and ammonium lignin sulfonate. A hemorrhagic diathesis developed after

the injection of large doses of polyanethol. Demole and Reinert concluded that the anticoagulating effect of these and similar macromolecular agents (heparin, hirudin, novirudin, germanin, Witte's peptone and other polypeptides) was not specific but due to the macromolecular character of these substances.

Of much greater importance in this respect are the observations made in connection with the parenteral introduction of solutions of acacia (Jackson and Frayser; Dick, Warweg and Andersch; Onozaki and Sanada; Staub and co-workers; Fukuhara and Hiratsuka; Hall and Gibson; Frimberger; Heckel and co-workers; Yuile and Knutti; Christie, Phatak and Olney; Lucia and Brown; Goudsmit, Power and Bollman; Studdiford; Peoples and Phatak; Hueper), polyvinyl alcohol (Hueper; Hueper, Landsberg and Eskridge; Martin and Hueper), pectin and methyl cellulose (Hueper) into various animals (rabbits, dogs, rats). Solutions of these agents intravenously introduced remain in the blood over long periods, forming with the plasma finely dispersed emulsions. The immediate effect produced on the blood by these macromolecular agents consists of a sharp and marked but transitory drop in the number of circulating leukocytes (colloidoclastic leukopenia), the result of a reduction in the number of the polymorphonucleated leukocytes. When larger amounts of a hypertonic macromolecular solution are administered, there is found an appreciable decrease also in the number of erythrocytes and in the amount of hemoglobin, caused by the ensuing hydremia. The erythrocytic conglutination and sedimentation are considerably accelerated, and the coagulation of the blood after a brief period of hastened clotting following immediately on the injection of the macromolecular matter is usually markedly slowed. These reactions are accentuated when repeated injections are made. Often there develops, however, a moderate to marked absolute increase of the immature myeloid cells, sometimes accompanied by considerable leukocytosis. The platelets after the administration of polyvinyl alcohol and acacia show a marked drop; after the injection of methyl cellulose the number of these elements remains unchanged or is even moderately increased. Similarly, the prothrombin time remains within normal limits when polyvinyl alcohol or acacia is administered, whereas there is a lengthening of the prothrombin time after the introduction of methyl cellulose. Not infrequently a hemorrhagic diathesis is observed after the prolonged injection of any of these three substances. The viscosity of the blood and especially that of the plasma can be increased more than fivefold above the normal level by prolonged administration of these agents in markedly hypertonic solutions. The average erythrocytic cell size and the osmotic resistance of these cells fluctuate within normal limits.

Blood chemical studies show that there occurs after the injection of a hypertonic solution of any of these agents a transitory but prolonged increase in the total plasma volume. With continued administration there develops a progressive reduction in the fibrinogen and total plasma protein contents of the blood, which may persist over long periods after the cessation of treatment with the macromolecular substances. While the oxygen-carrying power of the blood remains unchanged, there develops a reduction in the speed of oxygenation of the erythrocytes in the blood of dogs subjected to medication with polyvinyl alcohol, methyl cellulose or acacia (Christie, Phatak and Olney; Martin and Hueper) which is similar to that observed in rabbits having hypercholesteremia because of prolonged ingestion of a diet rich in cholesterol. This effect is attributed to the formation of surface films of the macromolecular matter on the erythrocytes which impairs the gaseous exchange across the interface. Inasmuch as chemical investigations or staining reactions reveal the presence of appreciable amounts of the exogenous macromolecular substances in the blood of the treated animals, the reported data indicate that nonproteinic macromolecules not only may substitute for proteinic ones in the maintenance of the colloidal osmotic pressure of the plasma but may influence by their prolonged presence in the plasma the production equilibrium of the plasma proteins.

The experimental and clinical evidence presented shows that a disturbance of the macromolecular colloidal equilibrium of the plasma caused by a relative or an absolute imbalance in favor of molecules equaling or surpassing in size those of the globulin portion of the plasma or by the presence of colloidally dispersed micellar aggregates elicits a number of blood changes which may be designated as the macromolecular hematologic syndrome (anemia, reduction of hemoglobin, leukopenia or leukocytosis, increase in conglutination and sedimentation of erythrocytes, impairment of coagulation, thrombopenia, hemorrhagic diathesis, decrease in fibrinogen and globulin [except in cases of hyperglobulinemia] and possibly also impairment of the speed of oxygenation of erythrocytes). The reactions characterizing the macromolecular hematologic syndrome are in part due to the disturbance caused in the physicochemical status of the plasma; in part they are caused by a direct interference of the pathogenic macromolecular material with the vital activities of certain organs engaged in the production of cellular elements and chemical constituents of the blood.

## ORGANIC REACTIONS

The pathogenic megalomolecular substances circulating in the plasma or formed locally in the tissues do not pass readily through the normal filtration membranes, i.e., the capillary walls, and are therefore not rapidly eliminated through the usual channels (kidneys, intestine and

other organs). Because of their comparatively marked chemical inertness, they resist speedy metabolic degradation and either are phagocytosed by various types of cells, including those normally not engaged in such work, or infiltrate, after causing some primary cellular injury, interstitial tissue spaces, giving rise thereby to intracellular and extracellular storage. Through the coalescence of dispersed particles in the blood or tissue fluids or through precipitation out of solution because of unfavorable environmental conditions or because of chemical or physical combination with other substances, they may clog capillary lumens as well as form films on interfaces of various kinds and thus interfere with the exchange of liquid and gaseous metabolites. As a result of these processes, the following lesions are observed:

*Brain.*—While the central nerve tissue is normally free from histochemically demonstrable glycogen, in an appreciable percentage of cases of glycogenosis this macromolecular substance is found within the ganglion cells of the brain and spinal cord, leading to swelling and morphologic distortion of these cells (Kimmelstiel; Siegmund; Wagner; Antopol) and appearance of cerebral symptoms. The presence of similar but more extensive anatomic lesions as well as of severe nervous symptoms is well known in connection with the two lipoidoses of infancy, Tay-Sachs amaurotic idiocy and Niemann-Pick disease, which result from accumulation of lipoidal material in these cellular elements (ganglion cells and glial foam cells).

Morphologic changes affecting both the ganglion cells and the glia cells and resembling closely those seen in the lipoidoses occur occasionally in animals (rabbits, rats) subjected to prolonged treatment with polyvinyl alcohol. Animals of the same species as well as dogs treated with methyl cellulose often show an accumulation of foam cells in the choroid plexus. The presence of polyvinyl alcohol in the distorted and huge, swollen nerve cells is readily demonstrated in sections stained with compound solution of iodine, which show the blue-colored polyvinyl alcohol content of these cells. The presence of methyl cellulose in the foam cells of the choroid plexus can be surmised by the negative reaction obtained with fat stains, such as sudan III. The conditions possibly existing in this respect in connection with prolonged administration of acacia (arabinosis) have not yet been adequately studied.

*Cardiovascular System.*—Glycogenosis is characterized, especially in the cardiomegalic type, by excessive accumulation of glycogen in the muscle cells of the heart, which are transformed thereby into swollen, vacuolated and distorted cells. These cells apparently undergo ultimately degenerative changes, as small highly vascularized, hyalinized and fibroblastic foci are occasionally found in large numbers in the myocardium, particularly in the interventricular septum (Antopol), while their functional deficiency is attested by the circulatory failure

finally causing death of the subject. Glycogen is also accumulated in the muscular walls of arteries and veins, producing a vacuolar status of the media, and is found within the reticuloendothelial cells in severe glycogenosis (Siegmund).

In Niemann-Pick lipoidosis, interstitial foci of foam cells occur in the heart.

Amyloid is deposited primarily within and around the walls of small vessels and capillaries of various organs (liver, spleen, kidneys and others) and within the reticulum of the sinusoidal structures of the splenic pulp and of the lymph nodes in addition to being laid down in the endocardium. In experimental amyloidosis, amyloidotic swelling and fusion of the glomerular endothelium precede the extravascular precipitation of this material in the tufts (Grayzel and co-workers) and thus the ultimate functional and morphologic destruction of these parts of the kidney. Dick and Leiter commented on the highly excessive incidence of aortic medial necroses, calcifications and atheromatous lesions in rabbits with experimental amyloidosis.

In this connection mention may be made of an observation of Masugi and Sato, who after repeatedly injecting horse serum intravenously into rabbits noted that some of the glomerular tufts were enlarged and filled with a homogeneous plasmatic mass while others were ischemic and adherent to the capsules and contained an increased number of nuclei. Proliferative and degenerative endothelial changes of the renal glomeruli characteristic of glomerulitis and glomerulonephritis are observed after the injection of a nephrotic immune serum into sensitized animals (Horn) and may represent reactions to the precipitation of macromolecular antigen-immune bodies in these vascular structures. The presence of a causal mechanism of this type is suggested by the fact that in rabbits the parenteral injection of foreign proteins (egg albumin, kidney and liver autolysates, squash seed globulin and others) results in excretion of the injected antigen over periods of several months, indicating retention of this material in the body in an essentially unchanged form (Briggs; Spinka and Weichselbaum; Harvey). The investigations of Bourdillon, on the other hand, showed that nephritic glomeruli exhibit graded permeability by withholding the larger molecular blood proteins (albumins and globulins) while letting the lighter protein fractions pass into the urine.

It is of significance in this connection that Heinlein after repeatedly introducing macromolecular colloidal substances, such as blood serum, into the blood stream found vascular changes (swelling and palisade arrangement of endothelial cells, loosening of media and perivascular mononuclear infiltration and eosinophilia) such as are observed in hyperergic-allergic reactions, in which allergic immune bodies are precipitated on the intimal lining of the reacting vessels, giving rise

to endothelial proliferation and medial degeneration. Holman attributed the vascular necroses occurring in dogs treated with intravenous injections of canine plasma and with uranium nitrate to an allergic mechanism. Hueper observed extensive arteriosclerotic lesions in dogs which had received repeated intravenous injections of large amounts of aminoantipyrine azoprotein (egg albumin).

While the possibility of a causal relation between such allergic reactions and the subsequent development of arteriosclerotic lesions is still a debated matter, Hueper, working with exogenous macromolecules, recently showed that the prolonged presence of polyvinyl alcohol or of methyl cellulose in the blood of dogs, rabbits and rats leads readily and consistently in both the elastic and the muscular arteries to extensive atheromatous and arteriocalcinotic changes which are morphologically identical with those observed in ordinary cholesterol atheromatosis but which result from the interaction of the two exogenous macromolecular substances with the vascular walls and their retention therein. In these investigations (Hueper; Hueper, Landsberg and Eskridge; Martin and Hueper) it was shown by histologic and biochemical methods that the polyvinyl alcohol, as well as the methyl cellulose, each present in the plasma in the form of a fine emulsion, tends to cover the intima and the surfaces of the erythrocytes with a film that interferes with the proper exchange of gaseous and nutritive matter and waste products between the blood and the tissues composing the vascular walls. The resulting vascular anoxemia and impaired nutrition leading to degenerative changes in the arterial walls are intensified by the phagocytosis of the exogenous macromolecular matter by the endothelial cells and the subsequent imbibition of these substances into the media. Thorough experimental studies concerning a possible arteriosclerotogenic effect of acacia were lacking until a recent date. However, "atheromatous lesions" in the aorta have been observed in nephrotic children subjected to prolonged treatment with solutions of acacia (Falkenstein and Jackson). Hueper observed medial necroses and calcifications and intimal thickenings in the aortas of dogs subjected for several months to intensive intravenous treatment with 6 to 25 per cent solutions of acacia.

The development of the experimental polyvinyl alcohol atheromatosis and of the methyl cellulose atheromatosis takes place while the plasma shows usually more or less markedly increased viscosity due to the introduction of hypertonic solutions of these two macromolecular agents. It seems to be of importance that similar physicochemical conditions prevail in man during the development of cholesterol atheromatosis, particularly that of juvenile type. The cholesterol, an equally chemically stable substance, resistant to enzymatic degradation and impermeable to many agents, is present in the lipemic plasma characterizing these

conditions (diabetes mellitus, myxedema, hypothyroidism, xanthomatosis) in a colloidally dispersed form (Hirsch). It was shown by Martin and Hueper that the blood of rabbits made hypercholesteremic by dietary measures displays a distinct reduction of the speed of oxygenation of the erythrocytes which is similar in type and probably also in mechanism to that exhibited by the blood of dogs that have been given polyvinyl alcohol or methyl cellulose. These observations indicate that atheromatous and arteriosclerotic vascular changes are attributable at least in part to a disturbance of the macromolecular equilibrium of the plasma or to the presence of colloidally dispersed chemically inert matter which interferes with the oxygenation and nutrition of the vascular walls. This conception receives additional support from the fact that patients with hypertension, a condition often preceding or accompanying the development of arteriosclerosis, exhibit not infrequently an increased viscosity of the plasma which is caused by a relative shift in the ratio of the various plasma proteins in favor of the large molecular compounds (Harris and McLoughlin; Brundage, Cantarow and Griffith).

It is noteworthy that the capillary endothelium of the renal glomeruli participates in the retention of the parenterally introduced polyvinyl alcohol and methyl cellulose and is transformed thereby into swollen, balloon-like, clear cells as well as into multinucleated giant cells which cystically distend and ultimately obliterate the capillary lumens, leading to atrophy of these structures and their adherence to Bowman's capsules. In rabbits similar glomerular lesions are observed after prolonged and repeated intravenous injection of excessive amounts of markedly hypertonic solutions of acacia (Hueper; Horn), and in rabbits and dogs, of pectin solutions (Hueper). The large foam cell elements accumulated in the glomerular tufts in Niemann-Pick disease are thought to represent foam cell mononuclear histiocytes of the blood and are thus allegedly of different derivation.

While the reticuloendothelial cells of the splenic sinuses store polyvinyl alcohol, pectin, methyl cellulose and acacia, the Kupffer cells of the hepatic capillaries retain mainly polyvinyl alcohol and methyl cellulose, showing no or little affinity for pectin and acacia. Therefore, polyvinyl alcohol and methyl cellulose in this respect behave like the lipoids, whereas the two polysaccharides follow glycogen. It may be mentioned that morphologically similar lesions are observed in the reticuloendothelial cells of the livers and spleens of rabbits given intravenous injections of a colloidal solution of a polymerized silicic acid (Rössle).

The presence of polyvinyl alcohol and methyl cellulose in an unemulsified form in the plasma elicits intimal reactions of a foreign body type, consisting of granulomatous formations containing multi-

nucleated giant cells, foam cells, histiocytes and fibroblasts. These reactions are found in the pulmonary vessels in connection with the presence of polyvinyl alcohol (polyvinylosis) and occur in the testicular venous plexus in association with the presence of methyl cellulose (methylcellulosis). These lesions may completely obliterate the vascular lumens. Similar intimal reactions were observed by Rössle in the lungs of rabbits after the injection of colloidal silica and were seen by Hirsch following the intravenous introduction of such substances as non-emulsified lipids, cholesterol and fatty acids.

The hearts of animals that have been subjected to injections of polyvinyl alcohol (polyvinylized animals) sometimes contain perivascular foam cell infiltrations in the myocardium and show a thick foam cell lining of the left auricle, the wall of which may be permeated diffusely by foam cells containing polyvinyl alcohol.

*Hemopoietic System.*—Krakower reported the presence of large vacuolated cells in the spleen and bone marrow in cases of glycogenosis, leaving it open whether these storage cells contained glycogen or lipoids. The frequent involvement of the splenic pulp in amyloidosis, either as a perifollicular process or as a diffiuse reticular phenomenon, is one of the striking features of amyloidosis. Amyloid deposits are found in the bone marrow in connection with myelomatosis. In Gaucher's lipoidosis the reticulum cells of the spleen, lymph nodes and bone marrow are transformed into large, swollen, balloon-like cells with a honeycomb-like structure of the cytoplasm due to the retention of cerasin. Similar lesions occur in Niemann-Pick disease.

The introduction of the exogenous macromolecular carbohydrates—acacia, pectin, polyvinyl alcohol and methyl cellulose—into experimental animals and man leads to storage of these agents in the reticulum cells of the splenic pulp and of the lymph nodes, causing proliferation of foam cells, and in the case of polyvinyl alcohol, pectin and methyl cellulose, formation of numerous multinucleated giant cells. Highly excessive retention of these exogenous substances in this organ results in cellular degeneration and in local or diffuse parenchymatous necrosis in the enormously enlarged spleen. None of these macromolecular compounds, except pectin, is stored to any appreciable degree in the bone marrow. The bone marrow is usually a dense myeloid tissue, which during prolonged treatment with these compounds may contain numerous immature cells. While only minor amounts of polyvinyl alcohol, methyl cellulose and acacia are retained in the reticulum cells of the bone marrow, pectin is eminently stored by these cells and gives rise to diffuse foam cell transformation of these elements.

*Alimentary System.*—Glycogenosis is characterized usually by the presence of excessive amounts of glycogen in the liver cells, which are thereby changed into swollen foam cell structures, causing general

enlargement of the affected organ. In some instances there is retention of glycogen in the smooth muscle tissue of the pylorus, causing swelling and vacuolation of these cells (van Creveld; Siegmund; Wagner, and others).

Whereas amyloid is not deposited within any of the cells composing the alimentary tract, it is often found within the tissue spaces of the liver as well as in the interstitial tissue of the intestinal mucosa and submucosa. A foam cell transformation of the liver cells as well as accumulation of lipoid-containing foam cells in the intestinal submucosa is characteristic of Niemann-Pick disease.

Similar intestinal lesions occur in connection with polyvinylosis and methylcellulosis, the foam cells being filled in these cases by the endogenous macromolecular matter. Methyl cellulose, pectin and acacia are retained in the liver cells and cause foam cell transformation in them. Excessive storage of these substances leads to extensive local or diffuse necrosis of the hepatic parenchyma (Metcalf and Hawkins; Anderson and Givson), an effect which also occasionally follows extraordinary massive retention of polyvinyl alcohol in the Kupffer cells. Retention of polyvinyl alcohol thus follows more closely the pattern set by the lipoidoses, whereas retention of acacia, of pectin and of methyl cellulose resembles in this respect more the glycogenoses.

*Respiratory System.*—Nodular amyloid deposits within the wall of the tracheobronchial tree are rarely observed. Accumulations of lipoid-filled foam cells located within the alveolar spaces and in the interstitial tissue of the lung are found in Niemann-Pick disease. Similarly, interstitial foam cells containing polyvinyl alcohol or methyl cellulose are found in polyvinylosis and methylcellulosis. Accumulations of these cells, often showing multinucleated giant cells surrounding small foci of the extraneous macromolecular matter, are located most often in the perivascular and peribronchial tissues. It is remarkable that sometimes the alveoli of polyvinylized dogs are lined by a film or granules of polyvinyl alcohol and that mononuclear cells containing this substance occur in appreciable number under such circumstances in the alveolar and bronchial lumens. Similar foam cell foci were observed in the interstitial tissue of the lung by Rössle after the injection of colloidal silicic acid and presumably contained this material.

*Urogenous System.*—Accumulations of glycogen are frequently found in the tubular epithelial cells of the kidney in glycogenosis. Amyloid may be deposited in the interstitial tissue of the kidney, and lipoidal foam cells occur in these parts in Niemann-Pick disease.

The tubular epithelium in animals subjected to treatment with polyvinyl alcohol, methyl cellulose or acacia is often vacuolated and contains granules or globules of the foreign matter (Hueper; Hagerty). The interstitial tissue is often diffusely infiltrated with these agents,

which cause thereby a myxomatoid transformation of these structures. Numerous foam cells are present, often intermingled with multinucleated giant cells.

*Endocrine Organs.*—The accumulation of polyvinyl alcohol in the cortical cells of the adrenal gland and of polyvinyl alcohol, pectin and acacia in the reticuloendothelial cells of the medulla of the gland resembles closely the storage phenomena seen in this organ in hypercholesteremic xanthomatosis, Niemann-Pick disease and Gaucher's disease. The capillaries of the thyroid gland often contain casts of polyvinyl alcohol, while small droplets of this material are found in the colloidal content of the thyroidal follicles of polyvinylized animals. Polyvinyl alcohol occurs also in the form of a fine dust in the interstitial cells of the testis.

*Skin.*—Alopecia is sometimes observed in the presence of glycogenosis (Unshelm). A similar phenomenon occurs occasionally in dogs which have been given injections of polyvinyl alcohol. Ultimately they may become almost completely hairless. Histologic examination of the skin reveals the presence of polyvinyl alcohol globules in isolated cells and cell groups surrounding the hair follicles and thus apparently interfering with the proper nutrition of the cells of the hair bulb.

Polyvinyl alcohol intravenously or intraperitoneally introduced in large amounts may lead also to the development of cutaneous nodular deposits of this material, which is stored in the skin in foam cells and multinucleated giant cells, sometimes forming tuberculoid structures. These cutaneous deposits display a certain similarity in their appearance and in their localization at points of physiologic traumatization to xanthoma.

*General Effects.*—Progressive and marked retention of metabolically inert macromolecular matter of endogenous or exogenous origin leads eventually to a serious disturbance of the vital activities of the directly or indirectly affected cells and organs and thereby causes impairment of the general nutrition. This effect is reflected by the considerable loss of weight ultimately observed in glycogenosis (Unshelm) and in lipoidoses (Gaucher's disease) (de Lange) as well as in polyvinylosis, methylcellulosis (Hueper) and arabinosis (Jackson and Frayser). This development is accentuated by the lack of appetite observed during the advanced stages of these storage diseases.

## COMMENT

The evidence presented indicates that the presence within the blood and tissues of abnormal amounts of endogenous or exogenous, normal or abnormal macromolecular substances which are chemically relatively inert and which resist metabolic degradation and are therefore not

readily eliminated through the filtration membranes elicits a set of hematologic reactions and changes (the macromolecular hematologic syndrome) and causes the development of organic and particularly vascular lesions which display a certain degree of similarity in location and character (macromolecular diseases). The molecular size and the physical qualities (colloidal dispersion, viscosity, film formation, precipitability) appear to be more important than the chemical properties in determining the biologic reactions to these substances.

The macromolecular hematologic syndrome consists of the following signs and symptoms: a reduction in the number of erythrocytes, in the amount of hemoglobin and in the volume of packed blood cells; initial transitory leukopenia and lymphocytosis; persistent secondary myeloid leukocytosis; accelerated conglutination and sedimentation of the erythrocytes; thrombopenia or thrombocytosis; impaired coagulability; lengthened bleeding time; hemorrhagic diathesis; reduction of fibrinogen and total plasma protein content (except in cases of a hyperglobulinemic disturbance).

The organic manifestations are characterized by storage phenomena in various organs leading to organic enlargement and to formation of parenchymatous and histiocytic-endothelial foam cells. Degenerative vascular changes, often resembling or identical with atheromatous or arteriosclerotic lesions, are found in the majority of the macromoleculoses discussed.

While many of these reactions do not require explanatory comment, there are some responses in which the causative mechanism is not self evident.

Among the various hematologic reactions, the reduction in the number of erythrocytes, observed as an acute as well as a chronic effect, belongs to these causally more complex phenomena. The primary decrease in the number of these cells after intravenous injection of massive amounts of the colloidal macromolecular agent is in part attributable to direct as well as to osmotic dilution of the plasma; in part it may result from an arrest of conglutinated erythrocytes in the pulmonary capillaries (Hanzlik and Karsner). The secondary progressive erythropenia following prolonged introduction of the macromolecular substances is in part the product of osmotic hydremia; in part it is evidently the result of an arrest in erythropoiesis or in erythrocytic emission. While anemia might result from an accumulation of macromolecular substances in the bone marrow sufficient to interfere with the erythropoietic activities of this tissue, and whereas such a mechanism certainly accounts to some degree for the anemia associated with lipoidoses, the scarcity or absence of the macromolecular carbohydrates and proteins in the myeloid tissue does not favor this view. It seems to be possible, on the other hand, that the marked proliferation

of the reticulum cells of the spleen observed in the macromolecular storage diseases exerts an inhibitory effect on the erythropoietic function of the bone marrow. Such a relationship has been suggested by observations made in connection with the anemia found in disorders accompanied by hypersplenia (Banti's disease, Hodgkin's disease, tuberculosis of the spleen and others) (Schousboe; Engelbreth-Holm; Schmidt; Bock and Frenzel, and others). Attention may also be called to the fact that hyperactivity of the splenic reticulum may impair emission of leukocytes from the bone marrow and may thus account for the more persistent leukopenic reactions seen in macromolecular diseases.

The marked delay or complete inhibition of the coagulation of the blood characterizing disturbances of the macromolecular equilibrium of the plasma cannot be satisfactorily explained with the evidence available. Inasmuch as there are no appreciable changes in the prothrombin time and in the blood calcium level, and as there are no consistent abnormalities in the number of platelets and in the amount of fibrinogen, the impairment in the clotting qualities of the blood is due either to an interference with the precipitation of the fibrinogen or to the dilution of the plasma with a macromolecular matter which prevents the formation of a fibrinous network and thus causes the development of a soft, friable mushy clot. This development is accentuated by the rapid separation of blood cells and plasma resulting from the highly accelerated sedimentation and conglutination of the erythrocytes.

The more or less marked, prolonged or persistent reduction in the amount of fibrinogen and plasma protein characterizing many of the diseases due to exogenous macromolecular substances reflects the existing hepatic injury interfering with the production of these blood constituents. It is possible, however, that this lag may be in part an apparent one, as the extraneous macromolecules can serve as suitable substitutes of the proteinic compounds in maintaining the colloidal equilibrium of the plasma and may thereby influence the production of the normal plasma proteins by the liver (Madden and Whipple).

The evidence available suggests that the pathogenic macromolecular substances formed in the cells or taken up by them by phagocytosis, infiltration or absorption may become first an integral part of the cellular structures. With increasing accumulation of such compounds in the cells, from whose confines the macromolecular matter cannot readily escape unless the cellular membranes become defective because of degenerative or necrotic changes, the macromolecular material is laid down in the cells as a deposit or is liberated from its original organic bindings by the process of phanerosis. There exists also some evidence indicating that prolonged storage of such compounds within the cells is accompanied by increased polymerization, resulting in

changes of the physicochemical properties, such as solubility, viscosity, dispersion and reactivity to oxidative, reductive, hydrolytic and enzymatic influences. These alterations in turn increase the difficulties which are connected with the disintegration and removal of these macromolecular substances from the organism. With the ultimate degeneration and necrosis of the cells loaded with macromolecular material, this matter obtains extracellularity and may merge with that fraction of the macromolecular deposits which has assumed this status through direct imbibition on the part of the interstices and intercellular structures.

Experimental and clinical observations indicate that the organism is capable of removing slowly macromolecular deposits as long as they have not become too extensive and do not interfere therefore too seriously with the vital activity of the affected organs. Such removal takes place for instance through the kidney, and there are indications that the intestinal and bronchial mucosa may take part in the process, which is supported by the emigration of phagocytes and the desquamation of cells lining renal tubuli and pulmonary alveoli, loaded with the macromolecular matter, into the excretory lumens of the corresponding organs. Thus, there is a limited reversibility of macromolecular storage diseases and other macromolecular disorders. On the other hand, if the deposition of macromolecular substances persists at an excessive rate, or if the arrest of this process comes too late, after large and important parts of the affected organs have been destroyed or functionally put out of action, death ultimately results, for the changes produced have become irreversible.

## SUMMARY

The presence of retained endogenous or exogenous macromolecular compounds (proteins, carbohydrates, lipids) in the blood and the tissues gives rise to functional disturbances, physicochemical reactions and morphologic organic lesions which are directly related to, and characteristic of, macromolecular diseases.

The hematologic responses may be designated as the macromolecular hematologic syndrome (reduction in the number of erythrocytes and in the hemoglobin content, leukopenia or leukocytosis, increased conglutination and sedimentation of erythrocytes, impaired coagulation, thrombopenia, hemorrhagic diathesis, decrease in fibrinogen and globulin [except in cases of hyperglobinemia] and possibly also impairment of the speed of oxygenation of erythrocytes).

The organic changes caused by the intracellular and extracellular storage of the macromolecular material affects especially the various phagocytic cells of the liver, spleen and lymph nodes and the endothelial cells of the large and small blood vessels, including the glomerular

capillaries, as well as certain parenchymatous cells, such as the liver cells and ganglion cells of the brain.

Macromolecular compounds circulating with the plasma and interfering with the oxygenation and nutrition of the vascular walls by the formation of films give rise to atheromatous and arteriosclerotic lesions.

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# Notes and News

**Society News.**—The annual meeting of the American Association of Immunologists will be held in Boston, Wednesday and Thursday, April 1 and 2, 1942, in conjunction with the meeting of the Federation of American Societies for Experimental Biology, extending from April 1 to 4. The headquarters will be the Parker House.

The annual meeting of the American Society of Clinical Pathologists will be held in Philadelphia June 5, 6 and 7 at the Benjamin Franklin Hotel. A seminar on the reticuloendothelial system will be conducted by Dr. Paul Klemperer on Sunday, June 7.

The annual meeting of the American Association of Pathologists and Bacteriologists will be held at the Washington University School of Medicine, St. Louis, Thursday and Friday, April 2 and 3, 1942.

Because of the war, the thirty-second annual clinical congress of the American College of Surgeons will be held in Chicago, October 19 to 23, instead of in Los Angeles as originally planned. Headquarters will be at the Stevens Hotel. The twenty-fifth annual hospital standardization conference sponsored by the American College of Surgeons will be held simultaneously. The programs of both meetings will be based chiefly on wartime activities as they affect surgeons and hospital personnel in military and civilian service.

**University News, Appointments, Etc.**—Evarts A. Graham, professor of surgery in Washington University, St. Louis, has been awarded the Lister Medal for 1942 in recognition of his contributions to the science of surgery, and he will give the Lister Memorial Lectures in 1942, or later, under the auspices of the Royal College of Surgeons, England. Dr. Graham has been elected a member of the Royal Society of Sciences in Uppsala, Sweden.

Everett I. Evans, instructor in pharmacology and surgery at the Medical College of Virginia, Richmond, has been given a grant of $6,300 by the federal government through the Office of Scientific Research and Development, for further research on surgical shock.

Lewis R. Thompson, director of the National Institute of Health and assistant surgeon general in charge of scientific research, has been appointed chief inspecting officer of the United States Public Health Service, a new position in the Surgeon General's office. Rolla E. Dyer, chief of the division of infectious diseases, has been appointed to succeed Dr. Thompson as director of the National Institute of Health and as assistant surgeon general in charge of scientific research.

Lyman L. Daines, dean and professor of bacteriology and pathology in the University of Utah, died Dec. 12, 1941, of coronary occlusion, aged 58.

Joseph Rigney D'Aunoy, professor of pathology and bacteriology in Louisiana State University, has died at the age of 51.

Under the directorship of Michael J. Hogan, a laboratory for the study of the pathology of the eye has been opened at the University of California.

**Foundation News.**—According to the third annual report of the National Foundation for Infantile Paralysis $807,131.50 was spent as follows in 1941: research on viruses, $186,565.06; studies on nutrition, $67,969.17; research on

after-effects, $285,572.54; aid in epidemics and nursing and medical help in emergencies, $89,048.13; education, $154,091.11.

During its eighteenth year the Ella Sachs Plotz Foundation for the Advancement of Scientific Investigation made thirty-five grants. At present, research projects bearing on medicine and surgery will be favored. The maximum grant will usually be less than $500. Applications for 1942-1943 must be received before April 1942. The secretary is Dr. Joseph C. Aub, 695 Huntington Avenue, Boston.

**Cancer Bibliography.**—The National Cancer Institute has prepared copies of the bibliography which H. E. Eggers compiled in the preparation of his paper entitled "The Etiology of Cancer," published in the ARCHIVES OF PATHOLOGY (**12**:983, 1931; **13**:112, 296 and 462, 1932).

For the convenience of those who wish to consult this bibliography, copies have been deposited in the following libraries:

Boston Medical Library, 8 The Fenway, Boston.

Library of the College of Physicians, 19 S. 22d St., Philadelphia.

Library of the New York Academy of Medicine, 5th Ave. and 103d St., New York.

John Crerar Library, 86 E. Randolph St., Chicago.

Lane Medical Library, Sacramento and Webster Sts., San Francisco.

Library of the Surgeon General's Office, 7th and Independence Ave., S. W., Washington, D. C.

Library of the National Cancer Institute, Bethesda, Md.

It is suggested that a notice of this distribution of copies of the bibliography be inserted at the end of Dr. Eggers' paper in the ARCHIVES OF PATHOLOGY.

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## CORRECTION

In the article by Dr. Irving Graef entitled "Studies in Lipid Pneumonia: I. Lipid Pneumonia Due to Cod Liver Oil; II. Lipid Pneumonia Due to Liquid Petrolatum," which appeared in the November 1939 issue (ARCH. PATH. **28**:613, 1939), the headings "Dry Weight, Gm." and "Wet Weight, Gm." in table 2, on page 626, are transposed.

# Book Reviews

**Natural Resistance and Clinical Medicine.** David Perla, B.S., M.D., late pathologist and bacteriologist, Montefiore Hospital, New York, and instructor in medicine, Columbia University College of Physicians and Surgeons; and Jessie Marmorston, B.S., M.D., formerly bacteriologist to Montefiore Hospital; assistant in pathology, Cornell University Medical College. Pages 1,344. Price $10. Boston: Little, Brown & Company, 1941.

The part played by the makeup of the host in relation to disease has been the object of ever increasing attention in recent years. The results are scattered in thousands of publications in the literatures of all nations. The imposing volume of Perla and Marmorston is an attempt to boil down the mass of widely dispersed data. It can be stated at the outset that the job of condensation was successfully accomplished. The work is more than a mere compilation; it represents a critical and judicious analysis of a subject that is not only vast in its dimensions but extremely difficult in the handling because much of it is still in the making.

The material is divided into ten sections and forty-six chapters. The titles of the sections give a good idea of the subject matter: heredity and resistance (196 pages); age and resistance (171 pages); sex glands and resistance (83 pages); other endocrine glands and resistance (111 pages); humoral and cellular mechanisms and resistance (201 pages); liver, body surfaces and nervous system and resistance (73 pages); diet and resistance (244 pages); certain depression states and resistance (22 pages); climate and resistance (56 pages), and clinical aspects of resistance (72 pages). Each chapter ends with a critical summary and is followed by pages of references which contain the impressive total of over 5,000 items. The index is well organized.

Occasionally, unintelligible sentences are encountered; for instance, on page 14, "man is infected by three trypanosomes: Trypanosoma gambiense and Trypanocidal action of his serum, etc." Errors have crept in; on page 111 one reads ". . . the well-to-do Hundus . . . are less frequently afflicted . . . than are the Hindus . . ."; on page 197, ". . . the agglutinogens M.N and MN factors . . ."; on page 218, "Haeckle's" biogenetic law instead of Haeckel's; on page 558, a reduction in the "neutral bactericidal power" instead of natural bactericidal power; on page 1301 (index), "pneumococci infection." The statement on page 144 and again on page 197 that the isoagglutinins do not appear until after the second year of life may be questioned. German maps are reproduced on pages 96 and 99 without indication of the source.

The defects mentioned in the preceding paragraph do not detract from the value of this work. It is a fundamental contribution and a fitting monument to the memory of the senior author, whose untimely death interrupted a career rich in achievements and great in promise.

The book should prove a valuable source of information to all who are interested in resistance to disease.

The external appearance, the binding and the print are excellent.

# Books Received

JOSIAH MACY JR. FOUNDATION REPORT FOR 1937-1940. Pp. 125. New York: Josiah Macy Jr. Foundation, 565 Park Avenue, 1941.

THE MARCH OF MEDICINE. New York Academy of Medicine, Lectures to the Laity, 1941. Pp. 154. Price $2. New York: Columbia University Press, 1941.

REPORT OF THE PRESIDENT AND OF THE TREASURER FOR THE YEAR ENDED SEPTEMBER 30, 1941. Pp. 148. New York: Carnegie Corporation (522 Fifth Avenue), 1941.

THE 1941 YEAR BOOK OF PATHOLOGY AND IMMUNOLOGY. PATHOLOGY, edited by Howard T. Karsner, M.D., professor of pathology and director of the Western Reserve University Institute of Pathology, Cleveland. IMMUNOLOGY, edited by Sanford B. Hooker, A.M., M.D., professor of immunology, Boston University School of Medicine; member, Evans Memorial for Clinical Research and Preventive Medicine; immunologist, Massachusetts Memorial Hospitals. Pp. 623. Price $3. Chicago: The Year Book Publishers, Inc., 1941.

DIABETES MELLITUS. Zolton T. Wirtschafter, M.D., clinician in charge, clinic for diabetes, department of medicine, Mount Sinai Hospital, Cleveland; visiting physician, department of medicine, Cleveland City Hospital; clinical instructor in medicine, Western Reserve University School of Medicine, Cleveland. Morton Korenberg, M.D., former fellow, May Institute of Medical Research, The Jewish Hospital, Cincinnati; medical resident, Jewish General Hospital, Montreal. Pp. 186. Price $2.50. Baltimore: Williams & Wilkins Company, 1942.

SURVEY OF COMPOUNDS WHICH HAVE BEEN TESTED FOR CARCINOGENIC ACTIVITY. Jonathan L. Hartwell, research fellow, National Cancer Institute, National Institute of Health, United States Public Health Service. Pp. 371. Washington, D. C.: Federal Security Agency, United States Public Health Service, 1941.

THE NATIONAL FOUNDATION FOR INFANTILE PARALYSIS, INCORPORATED. ANNUAL REPORT, 1941. Pp. 58. New York: The National Foundation for Infantile Paralysis, Inc., 120 Broadway, 1941.

REPORT OF THE SECRETARY OF THE SMITHSONIAN INSTITUTION AND FINANCIAL REPORT OF THE EXECUTIVE COMMITTEE OF THE BOARD OF REGENTS, 1941. Pp. 136. Price $0.25. Washington, D. C.: United States Government Printing Office, 1941.